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THE EFFECT OF REPEATED DAILY EXPOSURE TO COLD AND OF IMPROVED PHYSICAL FITNESS ON THE METABOLIC AND VASCULAR RESPONSE TO COLD AIR

By W. R. KEATINGE

From the Medical Research Council Department of Experimental Medicine, University of Cambridge

(*Received* 19 *August* 1960)

Experiments made to determine the effect of field acclimatization to cold on men's metabolic response to cold have given conflicting results (Burton, 1941; Leblanc, 1956; Scholander, Hammel, Hart, LeMessurier & Steen, 1958). These might be explained in part by the observation that different types of quite brief exposure to cold in the laboratory may either decrease (Keatinge, 1959; Keatinge & Evans, 1961) or increase (Keatinge, 1960) the metabolic response to cold, probably through central nervous adjustments. The present experiments were therefore designed to show whether more prolonged exposure to cold caused similar changes in metabolic response, and whether physical training, which generally accompanies field acclimatization, alters men's subsequent metabolic response to cold. Changes in basal metabolic rate and in non-shivering heat production were also looked for, since there are reports that the basal metabolic rate of men may change with the season (Gessler, 1925; Brown, Bird, Boag, Boag, Delahaye, Green, Hatcher & Page, 1954; Osima, 1957) and cold acclimatization gives rats (Sellers, Scott & Thomas, 1954; Cottle & Carlson, 1956; Heroux, Hart & Depocas, 1956) the power to raise their metabolic rate in the cold without muscular activity. Muscle, skin and rectal temperatures were also measured to try to confirm or refute the theory of Carlson, Young, Burns & Quinton (1951) that cold-acclimatized men maintain higher skin and rectal temperatures in the cold at the expense of larger falls in the temperature of intermediate tissues.

METHODS

Subjects and procedure. The subjects were 14 naval ratings who volunteered for the experiments; it had been hoped to study 30 men but this number of volunteers could not be found. The men were given extra pay and leave as an inducement to volunteer for the experiments, which were made in January, February and March, 1960. Before the experiments the men had been carrying out routine duty in the British Isles, generally as ground personnel at Fleet Air Arm stations; this often involved some outdoor work, but never involved exposure to severe or prolonged cold. On the first 2 days they were given practice

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J. Physiol. **157**, 38–63.

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at wearing mouth-pieces and nose-clips and their height, weight, fat thickness, basal metabolic rate and the depth of the blood vessels in their index fingers were measured. The men then spent a day in the cold room. Before entering it they sat for 45 min wearing their normal clothing in a room at 30–33° C, at the end of which their skin and rectal temperatures were measured. They then removed most of their clothing, entered a cold chamber at 6° C ± 1° C wearing only short trousers, ankle socks and half-Wellington rubber boots, and sat down in the cold room with their fingers apart and resting on a wooden table top. A circulating fan gave air movement of about 30 cm/sec. At the end of 30 min their skin and rectal temperatures were measured again and during this 1st 30 min their expired air was collected, to determine their metabolic rate, and in some cases an electromyograph record was made. For the next 6½ hr the men remained in the cold chamber wearing shorts, socks and boots, sitting or standing and listening to a radio or playing cards. A hot lunch was brought to them during this time. They then sat still again with fingers spread out for 30 min while the measurements made in the morning were repeated and two 15 sec electromyograph records were made on each man. At the end of this 30 min when the men had been in the cold for 7½ hr, the temperature of the muscle of their forearms was measured with a needle thermocouple.

The men were then divided into 3 groups. During all except 2 of the next 19 days five of the subjects ('cold subjects') spent 7½ hr a day in the cold room, again wearing only shorts, socks and boots and sitting or standing; five men ('control subjects') read books or did light tasks indoors in a warm room; and four men ('working subjects') spent approximately 7½ hr a day doing a stepping exercise (18 cm, 24 cycles/min) indoors, or playing football or taking other strenuous exercise, often outside when the weather was fine. The 'working' subjects were always warm and were usually sweating while they were training; their metabolic rates were found to be 4·4–7·2 kcal/min during the stepping exercises. On the 10th and 11th of these 19 days, men in all groups were allowed to go home and do what they liked. At the end of the period of 19 days all the men spent a further day in the cold room, in which the experimental procedure of the first day in the cold was repeated. The cold subjects therefore spent 7½ hr/day in the cold room on 19 days out of 21. During the next 2 days the measurements made in the warm were repeated under the same conditions as before. All measurements on the cold, working and control subjects given the same numeral (e.g. 2*a*, 2*b* and 2*c*), were made simultaneously.

Metabolic determinations. Metabolic rates were determined from expired air, collected by mouth-pieces and nose-clips and passed into either Douglas bags or a Max-Planck Institute Respirometer. The oxygen and CO_2 content of the air was measured by an automatic analyser (Hartmann and Braun A/G, Frankfurt am Main); its volume was either read directly from the Respirometer or measured by blowing the Douglas bags through the Respirometer. The metabolic rate was calculated by the method of Weir (1949) based on the expired air volume corrected to s.t.p. and the difference between the oxygen concentration in the inspired and expired air. The CO_2 concentration in the cold room and its oxygen deficit were measured at the middle of each period of collection, and were of the order of 0·3–0·4 %. The error of the volume measurements was ± 1 % and that of the oxygen analyser was ± 2% of the difference between the oxygen concentrations in the inspired and expired air. For basal determinations the men went without breakfast and lay quietly in a dark room at 22° C for 45 min, before the 15 min collection of expired air was made from which their basal metabolic rate was determined.

Electromyograph records. A strip of waterproof adhesive plaster (Smith & Nephew, Ltd.) containing 2 circular holes, 7 mm in diameter and 4 cm apart, was placed across the upper part of the right forearm, the two exposed circles of skin scrubbed clean with soap and water, and contact made between each of them and a silver plate by a sponge soaked in saline. These plates were connected to the leads of an 'Elema' ink-jet electrocardiograph apparatus. In some experiments similar records were made over the biceps or the pectoralis major muscles instead of the forearm, as these showed more activity during shivering.

Temperature readings. Rectal temperature was measured by thermocouples inserted 11 cm and skin temperature by stirrup thermocouples. These thermocouples have been described elsewhere (Keatinge, 1959). Intramuscular temperature was measured by a thermojunction built into the tip of a No. 8 intramuscular needle. All the thermojunctions were welded and made from 28 gauge copper and constantan wire. The cold junction was in a flask of distilled water and ice, and the e.m.f. was led through a distributor box to a potentiometer whose readings were accurate to the nearest 0·05° C. Skin temperature readings were made at the base of the terminal phalanx of the left index finger, the flexor surface of the left forearm just below the elbow, and the left side of the back, 5 cm from the mid line at the level of the iliac crest. The intramuscular needle was inserted into the ulnar group of forearm muscles, 4 cm distal to the elbow and to a depth of 2·0 cm.

Thickness of epidermis. A sharp, hollow No. 16 needle was made to protrude through a flat layer of thick Perspex, the distance it protruded being adjusted by a screw thread. While the men's skin blood vessels were dilated in a warm room the device was pressed against the centre of the pad of the left index finger repeatedly with the needle protruding to progressively greater depths until a spot of blood appeared when the finger was squeezed. The depth at which the needle then protruded was taken as the depth of the blood vessels from the surface.

Weight, and subcutaneous fat thickness. The men were weighed wearing drawers only. Their skin-fold thickness was measured by 'Harpenden' calipers at the sites used previously (Keatinge, 1959).

RESULTS

The subjects. Table 1 shows the height, weight, and mean skin-fold thickness of the men. One man, 5*b*, was really obese both in appearance and as assessed by his skin-fold thickness, and subject 3*a* was also rather

TABLE 1. Height, fat thickness and weight of the subjects

Subject	Height (cm)	Mean skin-fold thickness (mm)	Weight (kg)
	Cold subjects		
1*a*	168	7·95	66·7
2*a*	163	10·33	59·9
3*a*	175	11·65	71·2
4*a*	174	9·40	73·7
5*a*	175	9·30	68·5
Mean	171	9·73	68·0
	Control subjects		
1*b*	166	7·23	59·0
2*b*	174	12·38	78·7
3*b*	181	6·73	73·5
4*b*	162	9·35	64·4
5*b*	171	21·40	88·9
Mean	171	11·42	72·9
	Working subjects		
1*c*	180	9·93	71·4
2*c*	177	5·48	65·1
3*c*	184	8·38	81·0
4*c*	173	8·10	67·8
Mean	179	7·97	71·3

fat both in appearance and as judged by his skin-fold thickness. Subject 2*c* was the thinnest man.

General observations. The men were generally unable to concentrate on a book for more than a few minutes at a time on their first day in the cold, but during the next 4–6 days in the cold the 'cold' subjects' subjective discomfort decreased and they were thereafter able to read for long periods. This subjective improvement was only relative, however, and all men still found a day in the cold unpleasant even at the end of the period of acclimatization. The subjective improvement was noticed most in the afternoons and the men generally found the first hour or two in the cold room at least as unpleasant towards the end of the 19 days as at the start. The men often complained of muscular stiffness, mainly in the shoulders, at the end of their first day in the cold. This ceased after a few days of acclimatization.

TABLE 2. Effect of acclimatization to cold and of improved physical fitness on metabolic rate during first 30 min in cold room (kcal/min)

Exposure to cold			Controls			Physical training		
Subject	Before	After	Subject	Before	After	Subject	Before	After
1*a*	1·73	2·02	1*b*	1·77	1·56	1*c*	1·95	1·75
2*a*	1·50	1·62	2*b*	1·70	1·78	2*c*	2·82	2·01
3*a*	1·66	1·32	3*b*	1·83	1·68	3*c*	2·22	1·75
4*a*	1·89	2·28	4*b*	1·63	1·84	4*c*	1·49	1·15
5*a*	1·83	1·68	5*b*	1·57	1·50			
Mean	1·72	1·78	Mean	1·70	1·67	Mean	2·12	1·67

$^{*}S\bar{d}$ 0·130, $P < 0·05$.

In Tables 2, 3, 6 and 8, $^{*}S\bar{d}$ refers to the difference between results before and after acclimatization.

Visible shivering and electromyographs. During the first 30 min of a day in the cold room the men appeared to shiver less after physical training than before it, but acclimatization to cold had no obvious effect on, and certainly did not reduce, the intensity of the men's shivering during their first 30 min in the cold. During the last 30 min in the cold room, improved physical fitness did not appear to alter the intensity of their shivering but the men appeared to shiver less after than before acclimatization to cold. Shivering was intermittent and no quantitative assessment of the electromyograph records has been attempted; the records merely confirmed the presence of muscular activity in the acclimatized men in the cold room.

Metabolic rates. Table 2 shows that improved physical fitness reduced the men's metabolic rate in the first 30 min in the cold room. Acclimatization to cold on the average slightly increased the metabolic rate at this time, although it decreased it in two men, one of whom (3*a*) was the fattest of the group, and the other of whom (5*a*) was noticed to be particularly anxious on the first occasion. Table 3 shows that acclimatization to cold

reduced the men's metabolic rate measured 7–7½ hr after they entered the cold room, but that improved physical fitness did not do so at this stage of the exposure to cold. Table 4 shows that neither acclimatization to cold nor improved physical fitness altered the men's basal metabolic rates.

TABLE 3. Effect of acclimatization to cold and of improved physical fitness on metabolic rate during last 30 min in cold room (kcal/min)

Exposure to cold			Controls			Physical training		
Subject	Before	After	Subject	Before	After	Subject	Before	After
1*a*	2·94	2·53	1*b*	2·37	1·84	1*c*	2·74	1·99
2*a*	2·68	2·22	2*b*	2·21	2·80	2*c*	2·95	3·14
3*a*	2·59	2·32	3*b*	2·62	3·04	3*c*	3·18	2·83
4*a*	3·40	3·32	4*b*	2·43	2·06	4*c*	2·26	2·90
5*a*	2·59	2·05	5*b*	2·38	2·19			
Mean	2·84	2·49	Mean	2·40	2·39	Mean	2·78	2·72

$\overline{Sd}$ 0·081, $P < 0·025$.

TABLE 4. Effect of acclimatization to cold and of improved physical fitness on basal metabolic rate (kcal/min)

Exposure to cold			Controls			Physical training		
Subject	Before	After	Subject	Before	After	Subject	Before	After
1*a*	1·13	1·29	1*b*	1·22	1·14	1*c*	1·37	1·31
2*a*	1·35	1·30	2*b*	1·33	1·45	2*c*	1·19	1·33
3*a*	1·18	1·03	3*b*	1·56	1·42	3*c*	1·46	1·31
4*a*	1·47	1·30	4*b*	1·26	1·47	4*c*	1·18	1·20
5*a*	1·24	1·45	5*b*	1·24	1·36			
Mean	1·27	1·27	Mean	1·32	1·37	Mean	1·30	1·29

TABLE 5. Rectal temperature just before entering the cold room (° C)

Exposure to cold			Controls			Physical training		
Subject	Before	After	Subject	Before	After	Subject	Before	After
1*a*	37·35	37·20	1*b*	37·45	37·50	1*c*	37·40	36·85
2*a*	37·15	37·30	2*b*	37·60	37·45	2*c*	37·15	37·15
3*a*	37·10	37·10	3*b*	37·50	37·05	3*c*	37·60	37·20
4*a*	37·40	37·85	4*b*	37·15	37·60	4*c*	36·75	37·10
5*a*	36·90	36·75	5*b*	37·60	37·35			
Mean	37·18	37·24	Mean	37·46	37·39	Mean	37·23	37·08

Principal temperature findings. Table 5 shows that improved physical fitness tended to lower the rectal temperature of the working subjects at the time they entered the cold room, though the change was not significant. Table 6 shows that acclimatization significantly increased the rate at which their rectal temperature fell during 7½ hr in the cold room. Table 7 shows that neither acclimatization nor physical training altered the temperature taken up by the men's fingers after 7½ hr in the cold room. This temperature was always under 14° C.

Tables 8 and 9 show that acclimatization to cold did not have any consistent effect on either the forearm skin or forearm muscle temperature after 7½ hr in the cold room; both were higher in some men after acclimatization, but in subject 3*a* both were substantially lower after acclimatization. However, improved physical training significantly increased the

TABLE 6. Effect of acclimatization to cold and of improved physical fitness on the fall in rectal temperature during 7½ hr in cold room (° C)

Exposure to cold			Controls			Physical training		
Subject	Before	After	Subject	Before	After	Subject	Before	After
1*a*	0·20	0·25	1*b*	0·85	0·90	1*c*	0·90	0·10
2*a*	0·35	0·40	2*b*	0·75	0·25	2*c*	0·10	0·00
3*a*	0·10	0·55	3*b*	1·00	0·80	3*c*	1·00	0·30
4*a*	+0·10	0·30	4*b*	+0·30	0·50	4*c*	+0·20	1·50
5*a*	+0·45	0·00	5*b*	0·65	0·15			
Mean	0·02	0·30	Mean	0·59	0·52	Mean	0·45	0·48

$\overline{Sd}$ 0·0943, $P < 0·05$.

TABLE 7. Effect of acclimatization to cold and of improved physical fitness on finger temperature after 7½ hr in cold room (° C)

Exposure to cold			Controls			Physical training		
Subject	Before	After	Subject	Before	After	Subject	Before	After
1*a*	11·1	10·3	1*b*	8·1	8·9	1*c*	12·9	10·1
2*a*	13·3	13·6	2*b*	12·6	10·7	2*c*	11·6	12·5
3*a*	10·6	10·2	3*b*	10·0	9·7	3*c*	9·1	11·8
4*a*	9·3	10·6	4*b*	9·4	12·1	4*c*	12·1	10·7
5*a*	11·9	9·3	5*b*	9·8	10·5			
Mean	11·2	10·8	Mean	10·0	10·4	Mean	11·4	11·3

TABLE 8. Effect of acclimatization to cold and of improved physical fitness on forearm skin temperature after 7½ hr in cold room (° C)

Exposure to cold			Controls			Physical training		
Subject	Before	After	Subject	Before	After	Subject	Before	After
1*a*	23·4	24·6	1*b*	24·2	25·4	1*c*	18·6	20·4
2*a*	24·5	25·5	2*b*	22·5	23·1	2*c*	22·0	24·5
3*a*	23·6	20·9	3*b*	23·0	23·6	3*c*	22·0	25·2
4*a*	21·1	24·1	4*b*	20·8	21·2	4*c*	20·4	22·0
5*a*	24·6	25·1	5*b*	20·1	20·0			
Mean	23·4	24·0	Mean	22·1	22·7	Mean	20·8	23·0

$\overline{Sd}$ 0·364, $P < 0·01$.

forearm skin temperature during this time, and on average increased the forearm muscle temperature.

Other results. The men's finger, forearm and back temperatures just before entering the cold room were always between 33 and 37·5° C; they were not significantly altered by acclimatization or physical training. There was no significant difference before and after acclimatization or

physical training in the rate at which the men's finger or forearm skin temperatures fell in the first 30 min in the cold room, although both were on average maintained better after physical training than before it. Neither acclimatization nor physical training significantly altered the men's back temperatures at any stage in the cold room or the rate at which their rectal temperatures fell in the first 30 min in the cold. Nor did they alter the depth of the blood vessels from the surface of the index fingers. Men in all groups tended to gain weight during the experiments, on average 0·5 kg for the control group, 1·3 kg for the cold group, and 2·0 kg for the working group. The increases were not significantly different.

TABLE 9. Effect of acclimatization to cold and of improved physical fitness of forearm muscle temperature after 7½ hr in cold room (° C)

Exposure to cold			Controls			Physical training		
Subject	Before	After	Subject	Before	After	Subject	Before	After
1*a*	30·8	31·4	1*b*	30·6	29·0	1*c*	26·3	29·3
2*a*	32·2	32·5	2*b*	31·1	28·8	2*c*	29·5	29·1
3*a*	31·8	27·9	3*b*	33·0	33·9	3*c*	34·4	35·0
4*a*	31·4	30·5	4*b*	—	(28·6)*	4*c*	—	(27·8)*
5*a*	31·5	32·2	5*b*	30·9	30·2			
Mean	31·5	30·9	Mean	31·4	30·5	Mean	30·1	31·1

* Figures in brackets not included in means.

DISCUSSION

Effect of physical training

It is clear that improved physical fitness reduced the men's early metabolic response to cold. There is no evidence how it brought about the change, but since the reduction was observed more than 12 hr after the last period of exercise it cannot have been due to acute fatigue. The possibility that the thickness of the epidermis increased, and reduced the response to cold by protecting the cutaneous thermo-receptors, is virtually ruled out by the measurements of the depth of blood vessels. Quite hard and prolonged physical training seems to be necessary to reduce the metabolic response, since a single 160 min period of moderate physical exercise (Keatinge, 1960) did not reduce the metabolic response of men to subsequent cold immersion. Since physical training generally accompanies field acclimatization to cold, it probably plays a part in changes produced by this. In particular, it could account for the reduced metabolic response observed by Leblanc (1956).

The men also maintained rather higher forearm skin temperatures, presumably as a result of higher peripheral blood flow, after physical training. This confirms the observation of Adams & Heberling (1958) that men maintained higher skin temperature after physical training, and

training was probably responsible for the reduced vasoconstrictor response to cold observed by Keatinge & Evans (1958) in men after 10 days training in snow-covered country; the possibility of this was pointed out at the time, since the cold stress did not seem severe while the exercise was. The higher peripheral blood flow may explain why improved physical fitness did not reduce the metabolic rate after 7 more hours in the cold room (Table 3); since physical training increased the peripheral temperatures, and so presumably the heat loss, the men must ultimately have required higher metabolic rates in the cold after than before physical training. This may explain why Scholander, Hammel, Andersen & Løyning (1958) observed higher skin temperatures and higher metabolic rates after acclimatization in a group of men living and working outside continuously in air near freezing point.

Effect of acclimatization to cold on the size of the metabolic response to cold

Reports about the effect of acclimatization to cold on the metabolic response to cold have given different results, even if one ignores experiments in which a substantial amount of physical training is likely to have complicated the acclimatization. Burton (1941) reported that while heavily-clothed men generally had a poor metabolic response to cold, heavily clothed men used to life in a cold climate had a larger one, and Keatinge (1960) observed that exposure of men to air at 5° C for 160 min increased their metabolic response to a 30 min immersion at 15° C later in the day. On the other hand the metabolic response of men immersed for 20 min in water at 15° C was reduced by repeated immersion at this temperature (Keatinge, 1959; Keatinge & Evans, 1961) and Scholander, Hammel, Hart *et al.* (1958) observed lower metabolic rates and larger falls of skin and rectal temperatures during the night in aborigines than in Europeans sleeping out in Australia, where night temperatures were low but day temperatures were high. These various findings could be explained as adjustments made by the central nervous system, not necessarily at conscious level, in the light of experience obtained during acclimatization. On this interpretation the metabolic response to cold is accelerated, presumably by a conditioned reflex, when the experience obtained during acclimatization suggests that the cold stress will be prolonged and will become more severe than the early sensory stimulus indicates, while the response is reduced if the experience during acclimatization suggests that the exposure will soon cease and that a large response is unnecessary. The increase in the early metabolic response to cold often observed, together with the decrease in metabolic rate at the end of the day always observed, after daily exposure to cold in the present experiments, can hardly be ascribed to anything other than central nervous adjustments of this kind.

This interpretation of changes in the metabolic response is in keeping with certain changes observed in other responses to cold; the heart rate and blood pressure response to immersion of a hand for 60 sec in ice-water, a painful cold stimulus, is reduced by repetition, probably through the central nervous system (Glaser & Whittow, 1957; Glaser, Hall & Whittow, 1959), and the respiratory and heart-rate, as well as the metabolic, responses of men to immersion for 20 min in water at 15° C are reduced by repeated immersion at this temperature (Keatinge & Evans, 1961).

Effect of acclimatization on skin temperature in the cold

Repeated exposure to cold did not increase the men's skin temperature in the cold. Glaser (1950) reported that men maintained their skin temperatures in the cold slightly better after repeated exposure to cold, although Stein, Eliot & Bader (1949) reported contrary evidence in men allowed to work occasionally during their exposure to cold. Mackworth (1952) has reported that repeated exposure of heavily clothed men to cold without exercise reduced their finger numbness in the cold, although the difference could only be detected if the tests were made under strictly limited conditions of cold stress. Krieder, Iampietro, Buskirk & Bass (1959), studying men living for 14 days in air at 15·6° C and not taking exercise, found that at night, when the men had some protection from a sheet and blanket, they tended to maintain higher skin and lower rectal temperatures after than before acclimatization, although their daytime skin and rectal temperatures did not change. It therefore seems probable that repeated exposure to cold can modify somewhat the vasoconstrictor response of man to a limited degree of cold, perhaps by central nervous adjustments like those postulated to explain the metabolic changes, but such vascular modifications appear to be comparatively small.

Theory of heat loss from a larger shell after acclimatization

Carlson *et al.* (1951) obtained indirect evidence that a group of men lost heat from a larger 'shell' after they were acclimatized to cold in the field and Carlson, Burns, Holmes & Webb (1952–3) obtained evidence from intramuscular temperature measurements on three men, who were made to live in a cold climate, which they considered to give some support to their theory. In the present experiments acclimatization failed either to increase the subjects' skin temperatures, to lower their intramuscular temperatures, or to reduce their falls in rectal temperature in the cold (after 7½ hr in the cold) as the theory predicts. The effect of physical training on the men's metabolic rate in their first 30 min in the cold, and on their forearm skin temperatures was, however, similar to that observed by Carlson *et al.* (1951) after acclimatization, and it seems possible that improved physical fitness

was responsible for these authors' findings. As pointed out by these authors, an increase in the body shell would confer certain advantages during brief exposures to cold. It may well be produced briefly by physical training and by some types of acclimatization to cold, but the present experiments do not suggest that such a change plays an important part in acclimatization.

Non-contractile heat production in the cold and in the basal state

The question of whether the metabolic response of warm acclimatized adult men to cold is entirely due to increased muscle tone and to shivering has been the subject of prolonged dispute, but on balance (Chatonnet, 1959) there seems little doubt that muscular contraction is responsible for at least the major part of their response. However, rats acclimatized to cold acquire the ability to increase their heat production in the cold without shivering or other muscular activity (Sellers *et al.* 1954; Cottle & Carlson, 1956; Heroux *et al.* 1956; Depocas, 1960). The present subjects apparently did not acquire this ability to an important extent, since changes in the intensity of their shivering were associated with similar changes in metabolic rate.

Nor did the men's basal metabolic rate change. The basal metabolic rate of Eskimos (Brown *et al.* 1954) and of people living in poorly-heated European (Gessler, 1925) and Japanese (Osima, 1957) houses may increase in winter. However there is doubt about the significance of the change, at least in Eskimos (Rodahl, 1952; Burton & Edholm, 1955), and Scholander, Hammel, Hart *et al.* (1958) reported only a small increase in the basal metabolic rate of Europeans during 6 weeks of severe field acclimatization to cold; neither months of intermittent exposure to cold (Leblanc, 1956) nor 1–2 weeks continuous exposure of Europeans to moderate cold (Horvath, Freedman & Golden, 1947; Iampietro, Bass & Buskirk, 1957) have been found to increase the basal metabolic rate. The present observations are in keeping with the last findings, and show that while the metabolic response of men to cold was changed by 3 weeks of quite severe daytime exposure to cold, their basal metabolic rate was not.

SUMMARY

1. A group of men wearing only shorts and footwear sat or stood in air at 5–7° C for 7½ hr a day on 19 out of 21 days. Another group was given physical training in warm conditions during this time, except on the first and last days, when they kept still in the cold room.

2. The basal metabolic rate did not change in either group, and no evidence was found that the men acquired any important means of

increasing their heat production in the cold other than by increased muscle tone and shivering.

3. Repeated exposure to cold generally increased the men's immediate metabolic response in the cold room, but it always decreased their metabolic rate at the end of a day in the cold and it increased the rate at which their rectal temperatures fell. These changes are attributed to central nervous adjustments.

4. Physical training in the warm reduced the men's immediate metabolic response to the cold room and caused them to maintain rather higher forearm skin temperatures.

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STRUCTURAL DIFFERENCES OF FAST AND SLOW EXTRAFUSAL MUSCLE FIBRES AND THEIR NERVE ENDINGS IN CHICKENS

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Kuffler & Vaughan Williams (1953*a*, *b*) have shown that in the frog 'slow' extrafusal muscle fibres occur, which undergo a sustained contracture when the nerve to them is stimulated, rather than a contraction or twitch. It has been shown in the frog that the 'slow' or tonic muscle fibres are of the Felderstruktur type and a single muscle fibre has several '*en grappe*' type nerve endings (diffuse, multiterminal, distributed or 'over-all' innervation), while twitch or 'fast' (phasic) muscle fibres are of the Fibrillenstruktur type and each, in general, has a single '*en plaque*' or end-plate type nerve ending (discrete or focal innervation) (Krüger,1949; Günther, 1949; Gray, 1957, 1958; Hess, 1960).

Krüger (1950) and Krüger & Günther (1958) have shown in a variety of species of bird that Felderstruktur fibres and '*en grappe*' endings are found in the anterior latissimus dorsi (exclusively) and the medial part of the gastrocnemius (primarily), while Fibrillenstruktur muscle fibres and '*en plaque*' terminations are found in the posterior latissimus dorsi (exclusively), the lateral part of the gastrocnemius (predominantly) and exclusively in the pectoralis major, extensor metacarpi radialis and the flexor metacarpi ulnaris. Ginsborg (1960), by intracellular recording from single muscle fibres in the chicken, found both 'slow' and 'fast' fibres in the biventer cervicis, only 'slow' fibres in the anterior part of the latissimus dorsi, only 'fast' fibres in the posterior part of the latissimus dorsi and the extensor carpi radialis profundus, and some 'slow' fibres in the semispinalis cervicis. In addition, he has deduced from his physiological experiments that multiple or diffuse endings, about 340 μ from each other, occur on the 'slow' muscle fibres.

It is attempted in the present investigation to study further the structure of the muscle fibres in chickens to see if morphological differences do indeed occur and, if so, to define these differences further. Also, it is attempted, by the use of the cholinesterase technique, to see the nerve

endings on these muscle fibres, to describe their structure and any differences which might occur in different muscles, and to give direct morphological evidence for the occurrence of diffuse multiterminal endings.

METHODS

Adult chickens, chickens of 2–3 months, and chickens varying in age from newly-hatched to about 1 week in age, were used. The muscles used were the biventer cervicis and both parts, anterior and posterior, of the latissimus dorsi. For study of the structure of the muscle fibres, the muscles or pieces of muscle were fixed in Dalton's or Susa's fluid for 1 hr in an extended state, dehydrated, and embedded in methacrylate. Thin sections were cut for viewing the muscle fibres in the electron microscope, thicker sections were used for the phase-contrast microscope.

For the cholinesterase technique, the muscles were fixed in 10% glyoxal in tap water treated with calcium carbonate, teased, and stained by a modified Koelle technique, as described previously (Hess, 1960). The teased preparations were incubated in acetyl or butyryl thiocholine iodide with or without previous treatment with di*iso*propylfluorophosphate (DFP). The teased preparations were mounted in glycerine.

RESULTS

The structure of the muscle fibres

In the frog the 'slow' extrafusal muscle fibres were seen to differ structurally from twitch fibres in the distribution of fibrils and a granular sarcoplasm, in the appearance of the Z-disk, and in the presence of lipid droplets (Hess, 1960). The 'fast' Fibrillenstruktur muscle fibre had lipid droplets, discrete fibrils each surrounded by sarcoplasmic granules, and presented a Z-disk running straight across the fibril. The 'slow' Felderstruktur muscle fibre had no lipid droplets. Its fibrils were more haphazard in arrangement and not always surrounded by sarcoplasm, but adjacent fibrils appeared rather to touch or join each other at some places along their length. In addition, the Felderstruktur fibres always had a Z-disk running a zig-zag course across the width of the fibril. In transverse sections of these kinds of muscle fibres viewed in the phase-contrast microscope, the Fibrillenstruktur fibre had a punctate appearance with each discrete fibril visible, while the fibrils of the Felderstruktur fibre appeared more haphazard, irregular and larger, as if several fibrils had joined together.

In the chicken from hatching to about 7 days of age it is difficult to see any obvious differences in transverse sections of the muscle fibres in the phase-contrast microscope because of the small size of the fibres. The differences exist, but are not as obvious as they are in the muscles of adult chickens. Similarly, in transverse sections of muscle fibres viewed in the electron microscope, the differences in the distribution of the fibrils in different muscles of the young chicken are present but not obvious. The contracture which the fibres of the anterior latissimus dorsi undergo during

fixation makes it difficult to obtain an exact transverse section. However, in longitudinal sections of muscle fibres from newly-hatched chickens to those of 7 days of age the differences in the muscle fibres are striking (Pl. 2*A*, *B*). The muscle fibres from the posterior part of the latissimus dorsi have lipid droplets, the individual fibrils are discrete and surrounded by a few sarcoplasmic granules, and the Z-disk is always seen running straight across the fibril (Pl. 2*A*). The muscle fibres of the anterior latissimus dorsi are quite different. Here no lipid droplets are present, the fibrils do not appear as individuals and are not surrounded by sarcoplasm but appear to join their neighbours at several places along their length, and the Z-disk takes a zig-zag course across the width of the fibril (Pl. 2*B*). In the biventer cervicis both kinds of muscle fibre can be found.

Chickens 2–3 months after hatching also present these obvious differences in structure (Pl. 2*C*, *D*). However, no lipid droplets were encountered in the anterior or posterior latissimus dorsi of chickens of this age.

Adult chickens also present this picture (Pl. 3). Here the muscle fibres are large and the muscle fibrils can be seen quite clearly. In Susa-fixed material cut in transverse section and viewed in the phase-contrast microscope, the differences in the arrangement of fibrils in the muscle fibres can be seen (Pl. 1). The muscle fibres of the posterior latissimus dorsi have a punctate appearance (Pl. 1*A*), while the fibrils of those of the anterior latissimus dorsi are larger and more irregular and haphazard in appearance (Pl. 1*B*). The adult chicken differs from the younger chickens examined in the occurrence of lipid droplets; both kinds of muscle fibre can have lipid droplets. As in younger chickens, the biventer cervicis has both kinds of muscle fibre (Pl. 3*A*, *B*).

Thus, the posterior latissimus dorsi consists *almost* entirely (see below, p. 224) of 'fast' Fibrillenstruktur muscle fibres, while the anterior latissimus dorsi is composed entirely of 'slow' Felderstruktur muscle fibres. The biventer cervicis has both kinds of muscle fibre; although counts were not made, the general impression is that one kind of muscle fibre is about as numerous as the other in the latter muscle. The presence of lipid droplets is apparently not a significant difference between 'fast' and 'slow' muscle fibres in chickens.

The nerve endings of the muscle fibres

The morphology of the endings. Staining of cholinesterase reveals the motor endings. The posterior latissimus dorsi of the newly-hatched to 7-day-old chicken has endings that are variable in shape (Pl. 4*D*). They can appear as a dark line or blob of material on the muscle fibre. Frequently the endings appear annular and form hollow circles. The endings can also be seen to be surrounded in many instances by a darker

endings on these muscle fibres, to describe their structure and any differences which might occur in different muscles, and to give direct morphological evidence for the occurrence of diffuse multiterminal endings.

METHODS

Adult chickens, chickens of 2–3 months, and chickens varying in age from newly-hatched to about 1 week in age, were used. The muscles used were the biventer cervicis and both parts, anterior and posterior, of the latissimus dorsi. For study of the structure of the muscle fibres, the muscles or pieces of muscle were fixed in Dalton's or Susa's fluid for 1 hr in an extended state, dehydrated, and embedded in methacrylate. Thin sections were cut for viewing the muscle fibres in the electron microscope, thicker sections were used for the phase-contrast microscope.

For the cholinesterase technique, the muscles were fixed in 10% glyoxal in tap water treated with calcium carbonate, teased, and stained by a modified Koelle technique, as described previously (Hess, 1960). The teased preparations were incubated in acetyl or butyryl thiocholine iodide with or without previous treatment with di*iso*propylfluorophosphate (DFP). The teased preparations were mounted in glycerine.

RESULTS

The structure of the muscle fibres

In the frog the 'slow' extrafusal muscle fibres were seen to differ structurally from twitch fibres in the distribution of fibrils and a granular sarcoplasm, in the appearance of the Z-disk, and in the presence of lipid droplets (Hess, 1960). The 'fast' Fibrillenstruktur muscle fibre had lipid droplets, discrete fibrils each surrounded by sarcoplasmic granules, and presented a Z-disk running straight across the fibril. The 'slow' Felderstruktur muscle fibre had no lipid droplets. Its fibrils were more haphazard in arrangement and not always surrounded by sarcoplasm, but adjacent fibrils appeared rather to touch or join each other at some places along their length. In addition, the Felderstruktur fibres always had a Z-disk running a zig-zag course across the width of the fibril. In transverse sections of these kinds of muscle fibres viewed in the phase-contrast microscope, the Fibrillenstruktur fibre had a punctate appearance with each discrete fibril visible, while the fibrils of the Felderstruktur fibre appeared more haphazard, irregular and larger, as if several fibrils had joined together.

In the chicken from hatching to about 7 days of age it is difficult to see any obvious differences in transverse sections of the muscle fibres in the phase-contrast microscope because of the small size of the fibres. The differences exist, but are not as obvious as they are in the muscles of adult chickens. Similarly, in transverse sections of muscle fibres viewed in the electron microscope, the differences in the distribution of the fibrils in different muscles of the young chicken are present but not obvious. The contracture which the fibres of the anterior latissimus dorsi undergo during

fixation makes it difficult to obtain an exact transverse section. However, in longitudinal sections of muscle fibres from newly-hatched chickens to those of 7 days of age the differences in the muscle fibres are striking (Pl. 2*A*, *B*). The muscle fibres from the posterior part of the latissimus dorsi have lipid droplets, the individual fibrils are discrete and surrounded by a few sarcoplasmic granules, and the Z-disk is always seen running straight across the fibril (Pl. 2*A*). The muscle fibres of the anterior latissimus dorsi are quite different. Here no lipid droplets are present, the fibrils do not appear as individuals and are not surrounded by sarcoplasm but appear to join their neighbours at several places along their length, and the Z-disk takes a zig-zag course across the width of the fibril (Pl. 2*B*). In the biventer cervicis both kinds of muscle fibre can be found.

Chickens 2–3 months after hatching also present these obvious differences in structure (Pl. 2*C*, *D*). However, no lipid droplets were encountered in the anterior or posterior latissimus dorsi of chickens of this age.

Adult chickens also present this picture (Pl. 3). Here the muscle fibres are large and the muscle fibrils can be seen quite clearly. In Susa-fixed material cut in transverse section and viewed in the phase-contrast microscope, the differences in the arrangement of fibrils in the muscle fibres can be seen (Pl. 1). The muscle fibres of the posterior latissimus dorsi have a punctate appearance (Pl. 1*A*), while the fibrils of those of the anterior latissimus dorsi are larger and more irregular and haphazard in appearance (Pl. 1*B*). The adult chicken differs from the younger chickens examined in the occurrence of lipid droplets; both kinds of muscle fibre can have lipid droplets. As in younger chickens, the biventer cervicis has both kinds of muscle fibre (Pl. 3*A*, *B*).

Thus, the posterior latissimus dorsi consists *almost* entirely (see below, p. 224) of 'fast' Fibrillenstruktur muscle fibres, while the anterior latissimus dorsi is composed entirely of 'slow' Felderstruktur muscle fibres. The biventer cervicis has both kinds of muscle fibre; although counts were not made, the general impression is that one kind of muscle fibre is about as numerous as the other in the latter muscle. The presence of lipid droplets is apparently not a significant difference between 'fast' and 'slow' muscle fibres in chickens.

The nerve endings of the muscle fibres

The morphology of the endings. Staining of cholinesterase reveals the motor endings. The posterior latissimus dorsi of the newly-hatched to 7-day-old chicken has endings that are variable in shape (Pl. 4*D*). They can appear as a dark line or blob of material on the muscle fibre. Frequently the endings appear annular and form hollow circles. The endings can also be seen to be surrounded in many instances by a darker

staining border of cholinesterase, which is apparently the sub-neural apparatus.

The anterior latissimus dorsi of the newly-hatched to 7-day-old chicken has endings that vary very much in shape (Pl. 4*C*). The endings appear as a line or sometimes two or just a few streaks of cholinesterase-stained material. Circular endings, as seen in the posterior portion of the muscle, have not been found in the anterior part of the latissimus dorsi. A darker-stained border, probably equivalent to a sub-neural apparatus, can also be seen on some endings, although not as frequently or as clearly as in the nerve endings in the posterior latissimus dorsi.

The adult chicken reveals a rather drastic transformation in the shape of its nerve endings, compared to the newly-hatched chicken. The end-plates of the posterior latissimus dorsi (Pl. 4*E*, *H*, *I*, *J*) sometimes appear as relatively long finger-like extensions of cholinesterase-stained material. In other instances a series of intensely stained droplets comprises the ending. The endings of the anterior latissimus dorsi (Pl. 4*G*) are typically '*en grappe*' in structure. They appear as clusters or groups of droplets of cholinesterase-stained material and are very variable in shape. The biventer cervicis has many muscle fibres with end-plates and many other fibres have '*en grappe*' endings (Pl. 4*A*, *B*, *F*). The posterior latissimus dorsi also has a few muscle fibres with '*en grappe*' endings (Pl. 4*K*). The end-plates of the posterior latissimus dorsi are easily differentiated from the '*en grappe*' endings of the anterior latissimus dorsi after cholinesterase staining. The end-plates are stained more intensely, occupy a greater extent of the muscle fibre, and as will be seen below, occur singly on a muscle fibre, whereas the '*en-grappe*' endings are stained relatively lightly, consist of only a few droplets or a small cluster, and several endings always occur on a single muscle fibre.

All chickens of all ages have both true and pseudo-cholinesterase in their motor nerve endings, whichever the kind of ending. Incubation in acetyl thiocholine iodide after treatment with DFP reveals all nerve endings stained somewhat less intensely than without DFP treatment. Incubation in butyryl thiocholine iodide also reveals all nerve endings, although all the endings, especially the '*en grappe*' type, are stained much more lightly. Incubation in butyryl thiocholine iodide after DFP treatment inhibits the staining of all nerve endings.

The distribution of the endings. End-plates and '*en grappe*' terminations were never found on the same muscle fibre.

In the newly-hatched to 7-day-old chicken individual muscle fibres are difficult to tease, and are about 10 μ in thickness. However, when individual fibres are teased, it is possible to see a difference in the distribution of the endings between the muscle fibres of the anterior and

posterior parts of the latissimus dorsi. The posterior latissimus dorsi, as far as can be determined, has one ending per muscle fibre, while the anterior latissimus dorsi has several endings per muscle fibre, distributed rather evenly along the muscle fibre at distances of about 250–350 μ (Pl. 4*C*). It is difficult to tease the small muscle fibres of the newly-hatched chicken for any great length and thus difficult to ascertain if only one ending occurs per muscle fibre in the posterior latissimus dorsi.

In the adult chicken the teasing of the muscle fibres becomes relatively easy, and the fibres are about 60 μ in diameter. Fibres of 1 cm or more in length were teased from the posterior latissimus dorsi and on none of these fibres on which an end-plate occurred was there more than one nerve ending. The few fibres from the posterior latissimus dorsi with '*en grappe*' endings always had several endings along their length (Pl. 4*K*). The endings of adjacent muscle fibres occur at about the same level.

It is relatively more difficult to tease the fibres of the anterior latissimus dorsi, even in the adult, probably because of the 'slow' characteristics of these fibres and their tendency to undergo a contracture. Fibres were teased for distances of about 8 mm. All fibres revealed several '*en grappe*' endings along their length, separated by distances of about 1000 μ from each other. The endings are separated from each other by rather regular distances. The endings of adjacent fibres also occur nearly at the same level on the muscle fibre.

The fibres of the biventer cervicis present an advantage for preparing teased preparations in that the 'slow' fibres, mixed in with twitch fibres, are rather easier to tease. Muscle fibres with '*en grappe*' endings were teased for distances of 1 cm or more. As an example, one fibre about 12 mm in length had 11 '*en grappe*' endings at distances of 1200 μ, 900 μ, 800 μ, 1000 μ, 1000μ, 900μ, 1200 μ, 1700 μ, 1000 μ and 1100 μ from each other, which indicates the rather regular disposition of '*en grappe*' endings along the 'slow' muscle fibres of the chicken (Pl. 4*A*, *B*).

DISCUSSION

Extrafusal muscle fibres are of two kinds and their similarities and differences may be generally summarized as follows: (*a*) 'slow' muscle fibres which undergo contracture and do not give rise to a propagated potential when repetitively stimulated are of Felderstruktur type and are innervated by small motor nerve fibres which form multiple '*en grappe*' endings on each muscle fibre; (*b*) 'fast' muscle fibres which undergo a twitch and have a propagated potential with every stimulus are of Fibrillenstruktur type and are innervated by large motor nerve fibres which form usually one '*en plaque*' ending on each muscle fibre. End-plates and '*en grappe*' endings never occur on the same muscle fibre. This

will be referred to as the general scheme of muscle fibre innervation in the ensuing discussion.

Fish muscle fibres deviate from this scheme. 'Fast' muscle fibres of the fish have 'over-all' innervation (Barets, 1955; Barets & Le Touzé, 1956; Takeuchi, 1959) or nerve endings at each end of the muscle fibre ('myoseptal' endings) (Barets, 1952; Barets, Fessard & Le Touzé, 1956). Nevertheless, these fibres are called 'fast' because they have 'aborted' action potentials which, although not identical to the twitch fibres of the frog, resemble the propagation of 'fast' frog fibres more closely than the 'slow' fibres of the frog (Barets & Le Touzé, 1956; Barets *et al.* 1956; Takeuchi, 1959). The 'slow' fibres of the fish conform more closely to the scheme, undergo contracture and have multiple endings (Barets, 1952; Barets & Le Touzé, 1956; Takeuchi, 1959). The 'slow' muscle fibres of the fish are innervated by small nerve fibres, the 'fast' fish muscles by large fibres (Barets, 1952, 1955). Fish muscle fibres differ in their internal structure. However, muscle fibres with the same internal structures can be found in both 'slow' and 'fast' muscles. It is rather in the distribution of muscle fibres of different internal structure in 'fast' and 'slow' muscles by which the morphological contrasts between these kinds of muscle can be seen (Barets, 1952).

Amphibian muscle fibres conform to the scheme, as has already been mentioned in the introduction. However, there are amphibian muscle fibres which have myoseptal endings (Mackay, Muir & Peters, 1960; Lewis & Hughes, 1960) and which presumably would also have membrane characteristics different from typical 'fast' or 'slow' fibres. Their internal structure is as yet unknown.

Chicken muscle fibres, as shown in the present study, conform very well to the scheme. The size of the nerve fibres innervating the 'fast' or 'slow' chicken muscle fibres has not yet been determined. Although preliminary studies of the nerve to the anterior latissimus dorsi indicate that there are many small myelinated fibres, a more crucial study of deafferented nerves is necessary before making any further statement on the size of motor nerve fibres innervating the 'slow' extrafusal fibres of chickens. This problem is further complicated by the occurrence of spindles and intrafusal fibres in the anterior latissimus dorsi of the chicken which have been seen to have multiple endings (Hess, unpublished observations) and which would presumably be innervated by the same size nerve fibres as those innervating the 'slow' extrafusal fibres. In addition, Ginsborg (1959) and Ginsborg & Mackay (1960) have reported that 'diffusely innervated (chicken) fibres are capable of responding to adequate stimulation with propagated action potentials'. Amphibian 'slow' fibres apparently do not react in this way. This difference might be accounted for by the distribution of the

multiple nerve endings, which are scattered and irregular and can be very close together in the frog (endings were seen with only 60 or 120 μ between them (Hess, 1960)), while in chickens of the age used by Ginsborg, the '*en grappe*' endings appear to be rather evenly distributed along the length of the muscle fibres (about 250–350 μ between endings), are perhaps less concentrated than those of the frog, and occur at about the same level on adjacent muscle fibres.

More work must be done before it can be determined if mammalian extrafusal muscle fibres fit into this scheme. The occurrence of muscle fibres of Felderstruktur or Fibrillenstruktur types in mammals has not yet been confirmed, although the discoverer of such muscle fibres in frogs and birds believes that they occur (Krüger & Günther, 1955). The situation is especially difficult since '*en grappe*' endings are said not to occur on the extrafusal muscle fibres of mammals (except in eye muscles, see Tiegs, 1953) and 'slow' extrafusal muscle fibres are not found physiologically in mammals (Kuffler *et al.* 1953*b*), although Krüger (1960) believes that they do occur and that the same correlations can be made in mammals as in frogs and birds. In addition, the small motor nerve fibres in mammals are said to go to intrafusal muscle fibres only (Kuffler & Hunt, 1952).

Intrafusal muscle fibres have also been investigated with this scheme in mind. Frog intrafusal fibres receive large or small motor fibres (Katz, 1949) and can have '*en grappe*' multiple terminations or end-plate type endings (Gray, 1957). The membrane characteristics of the frog intrafusal fibre, even those with multiple nerve terminations, resemble twitch fibres more closely than 'slow' fibres (Koketsu & Nishi, 1957*a*). The differences between 'slow' extrafusal fibres and intrafusal fibres might be accounted for by the multiple terminations on intrafusal fibres being less concentrated than on 'slow' fibres (Koketsu & Nishi, 1957*b*). Similarly, in mammals, intrafusal fibres with multiple motor terminations react physiologically with propagated potentials like 'fast' muscle fibres (Eyzaguirre, 1960). Intrafusal mammalian fibres receive large or small motor fibres and have end-plate endings or multiple endings (Boyd & Davey, 1959; Diete-Spiff & Pascoe, 1959; Hess, 1961). However, the multiple motor endings are restricted to the juxta-equatorial region of the intrafusal fibre adjacent to the equatorial region where the sensory innervation occurs (Hess, 1961). This restriction of multiple motor nerve terminations to a limited region of the muscle fibre might account for the physiological similarities of the intrafusal muscle fibre and a 'fast' muscle fibre. Krüger (1960) and Barker & Gidumal (1960) have found differences in the myofibrillar arrangement in mammalian intrafusal muscle fibres. The correlation between kind of muscle fibre and kind of nerve ending remains to be worked out.

The source or origin of the multiple terminations remains to be deter-

mined in many of the above cases. I have concerned myself here only with the occurrence of multiple motor endings. These endings could come from the branches of a single axon in a peripheral nerve and hence from a single cell in the spinal cord or the branches of axons of several nerve cells could end on a single muscle fibre (polyneuronal innervation). In some cases, a single axon has been seen to pass along a muscle fibre and make endings. In other instances, polyneuronal innervation has been proven. I have not dealt directly with this problem in this investigation.

The multiply innervated muscle fibre seems to make its appearance first in the phylogenetic series of animals. Invertebrate muscles are innervated in this manner (see Hoyle, 1957). The similarities of invertebrate muscles and those of fish, where even the 'fast' muscle fibres are multiply innervated, are striking. The presence of 'slow' multiply innervated fibres in frogs and chickens and the multiply innervated intrafusal fibres of frogs, chickens and mammals indicates that these multiply innervated fibres, 'slow' or 'fast' or classified in between these two extremes, are present throughout all animal groups. Just as 'the chief and most characteristic functions of nervous and other excitable tissues are performed by means of graded responses' and the 'all-or-none response is a special case of the general property of excitability' (Bishop, 1956), so it may be that the 'slow' multiply innervated muscle fibre appears first phylogenetically, undergoes modification in the distribution of its nerve endings and its ability to propagate an impulse and can be either 'fast' or 'slow' or in between, and is still present, highly modified, as the intrafusal muscle fibre in the highest animals.

SUMMARY

1. The extrafusal muscle fibres of chickens were studied in the electron and phase-contrast microscopes. Their nerve endings were examined in teased preparations after staining with the cholinesterase technique.

2. Fibrillenstruktur muscle fibres have regular fibrils, each surrounded by sarcoplasm and granules. The Z-disk runs straight across the width of the fibril. These are the twitch or 'fast' fibres.

3. Felderstruktur muscle fibres have irregular fibrils apparently joining with each other at certain points along the length of the fibrils, the fibrils are not regularly surrounded by sarcoplasm and granules, and the Z-disk appears zig-zag across the width of the fibril.

4. The presence of lipid droplets is apparently not a significant difference between 'fast' and 'slow' muscle fibres in chickens.

5. End-plate endings are the ordinary kind of ending and occur one per muscle fibre on Fibrillenstruktur muscle fibres only.

6. Several '*en grappe*' terminations occur on each muscle fibre and only on fibres of Felderstruktur.

7. The posterior latissimus dorsi of the chicken from hatching to 1 week of age, from 2 to 3 months of age, and adult, consists almost entirely of muscle fibres of Fibrillenstruktur which have one ordinary end-plate on each fibre. There are, however, a few fibres which receive several '*en grappe*' terminations in the posterior latissimus dorsi and are probably of Felderstruktur type.

8. The anterior latissimus dorsi of such chickens consists entirely of muscle fibres of Felderstruktur. Several '*en grappe*' terminations are found on each of these muscle fibres, occurring regularly along the length of the muscle fibre and separated by distances of about 250–350 μ in 1- to 7-day-old chickens, and by about 1000 μ in adults.

9. The biventer cervicis consists of mixed Fibrillenstruktur and Felderstruktur muscle fibres, the former with one end-plate type ending per muscle fibre and the latter with several '*en grappe*' terminations on each muscle fibre.

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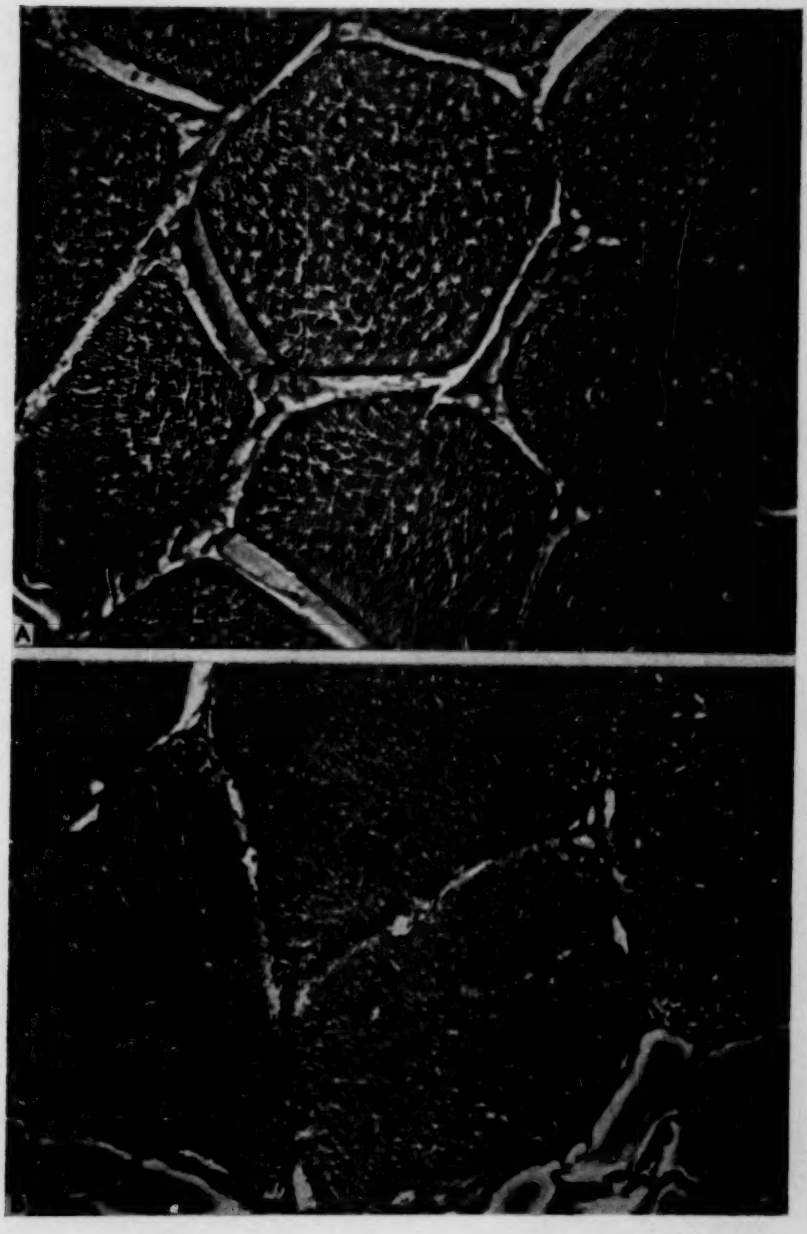
EXPLANATION OF PLATES

All photo-micrographs are from chicken muscles. The scale marks on the electron photomicrographs represent 1 μ; those on the light and phase-contrast photomicrographs represent 10 μ.

Pl. 1. Susa-fixed, embedded in methacrylate, transverse sections, phase-contrast micrograph. *A*. The posterior latissimus dorsi, Fibrillenstruktur. *B*. The anterior latissimus dorsi, Felderstruktur.

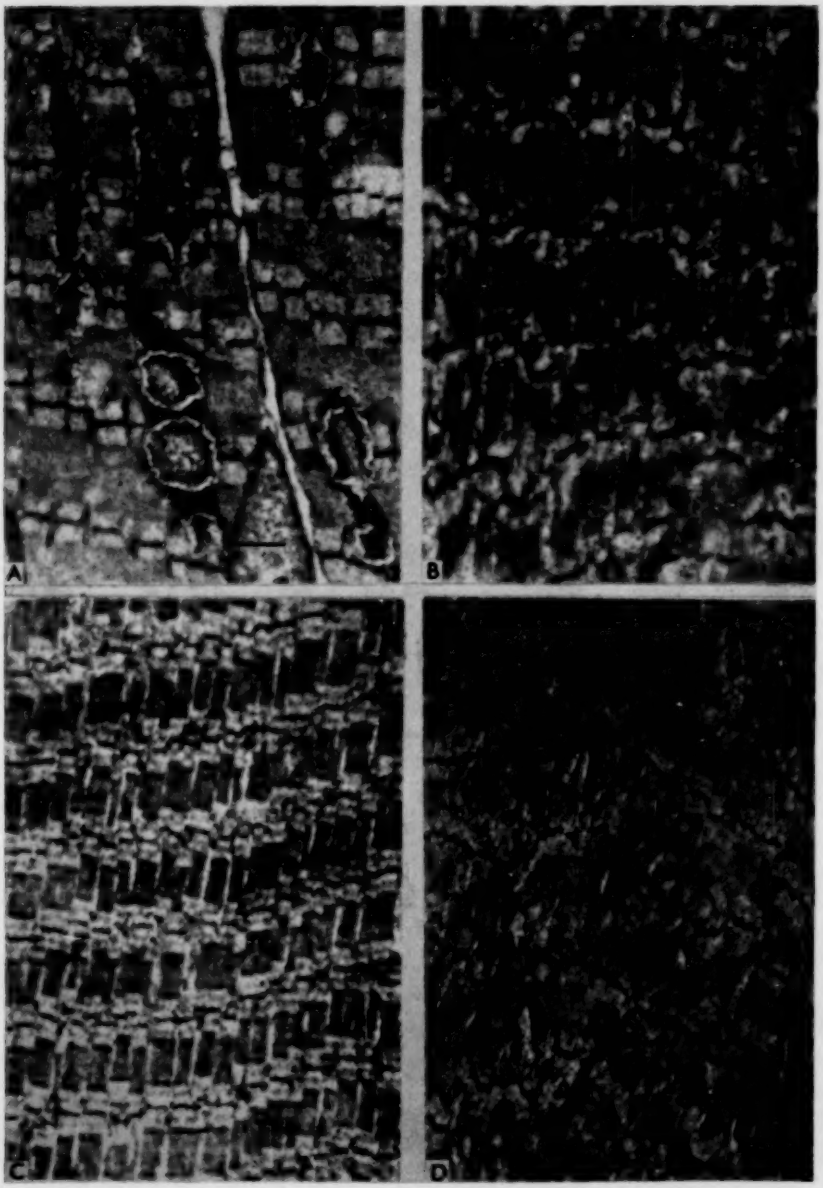
Pl. 2. *A*, *B*. Chickens from hatching to 7 days, longitudinal section, electron micrograph. *A*. The posterior latissimus dorsi, Fibrillenstruktur. *B*. The anterior latissimus dorsi, Felderstruktur. C, D. Chickens of 2–3 months, longitudinal section, electron micrograph. *C*. The posterior latissimus dorsi, Fibrillenstruktur. *D*. The anterior latissimus dorsi, Felderstruktur.

Pl. 3. *A*, *B*. Adult chickens biventer cervicis, longitudinal section, electron micrographs. A Fibrillenstruktur muscle fibre is on the top of each photograph; a Felderstruktur muscle fibre is on the bottom of each photograph.



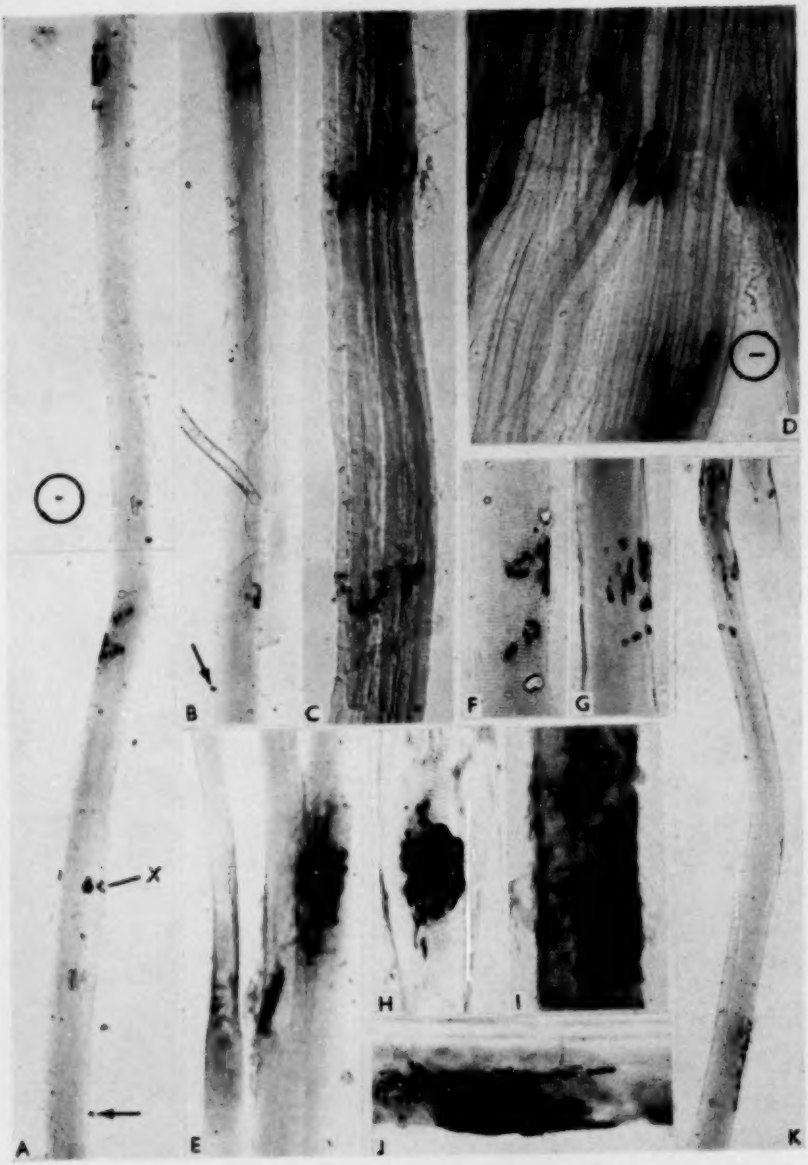
A. HESS

(*Facing p.* 230)



A. HESS

A. HESS



A. HESS

Pl. 4. Teased muscle fibres, cholinesterase stain. *A*, *B*, *E*, *K* are the same magnification; *C*, *D*, *F*–*J* are the same magnification. *A*, *B*. '*En grappe*' endings from the biventer cervicis of adult chicken. Montage; the fibre in *A* and *B* is continuous at the arrow. Four out of 11 '*en grappe*' endings on this fibre are seen. *X* is a piece of dirt, not an ending. *C*. '*En grappe*' endings from anterior latissimus dorsi of 3-day-old chicken. *D*. '*En plaque*' endings from posterior latissimus dorsi of 3-day-old chicken. *E*. '*En plaque*' endings from posterior latissimus dorsi of adult chicken. *F*. '*En grappe*' ending from biventer cervicis of adult chicken. *G*. '*En grappe*' ending from anterior latissimus dorsi of adult chicken. *H*, *I*, *J*. '*En plaque*' endings from posterior latissimus dorsi of adult chicken. *K*. '*En grappe*' endings from posterior latissimus dorsi of adult chicken.

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RENAL TRANSTUBULAR FLUX OF ELECTROLYTES IN DOGS WITH SPECIAL REFERENCE TO CALCIUM

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Increasingly it is being realized that the appearance of a given ion in the urine is the resultant of various fluxes across the many membranes making up a nephron. Some of the evidence for the existence of ion fluxes in the kidney is as follows: Oken, Whittembury, Windhager, Schatzmann & Solomon (1959) were able to demonstrate the existence and to measure the extent of sodium flux into ('influx') and out of ('efflux') the proximal tubule of *Necturus*. Using an approach that has served as a basis for the study reported here, Chinard & Enns (1955) demonstrated tubular influx of sodium, concluding from their observations that most of the sodium and chloride excreted in the urine had been added to the urine by a transtubular route (see also Chinard, 1957). That portions of the renal tubule are permeable to ^{24}Na and ^{42}K has recently been confirmed by Morel & Falbriand (1959), who used a combination of stop-flow and isotope techniques. Earlier, Hoshiko, Swanson & Visscher (1956) had demonstrated that sodium and potassium move rapidly across the renal tubular cells of the bull frog.

Whereas it is uncertain whether transtubular sodium flux constitutes secretion in the conventional sense, potassium secretion is known to occur (Smith, 1951), some investigators (Morel, 1955; Davidson, Levinsky & Berliner, 1958) maintaining that the potassium in urine is largely derived from potassium secreted in the distal portion of the nephron.

The renal handling of the divalent cations calcium and magnesium, and that of the associated phosphate anion(s), on the other hand, has been explored much less. The general topic of calcium excretion in urine is reviewed by Irving (1957). Recently several workers (Howard, Wilde & Malvin, 1959; Wesson & Lauler, 1959; Samiy, Brown, Globus, Kessler & Thompson, 1960*a*) have reported that calcium is reabsorbed in a far distal area of the nephron of the dog, but the magnitude of proximal reabsorption of the electrolyte could not be determined in any of these experiments.

The present report offers evidence for bidirectional flux of calcium across the renal tubular epithelium. Further, it describes experiments

designed to relate the excretion pattern of calcium to those of potassium, sodium, strontium, magnesium, phosphate and exogenous creatinine in an attempt to define regions of transtubular calcium movement. Finally, it reports measurements of the specific activities of kidney tissue, blood plasma and urine, made in an attempt to relate tissue uptake and turnover of the injected isotopes to the observed excretion patterns. A preliminary account of this work has already appeared (Bronner & Thompson, 1959).

METHODS

Male or female mongrel dogs weighing 15–20 kg were anaesthetized by the intravenous injection of sodium pentobarbital, 30 mg/kg body weight. Through an incision made in the left flank the left renal artery was approached retroperitoneally with just sufficient dissection about it to identify its origin clearly. An incision was then made along the mid line of the lower abdomen, the urinary bladder was located and the ureters identified at the point where they entered the bladder wall. A small nick was made in each ureter and a small polyethylene catheter (1·19 mm internal diameter) inserted into each, advanced about 4 cm and tied securely. The length of each catheter and the distance it was inserted were kept identical in each individual experiment, so that catheter delay would be similar on the two sides. An osmotic diuresis was initiated by the infusion of 10% mannitol in NaCl solution, 0·9 g/100 ml., at a rate of approximately 10 ml./min. In most experiments the urine flow was elevated to about 6 ml./min from each kidney before experimental collections were begun. The polyethylene catheters were then attached to a ratchet-driven fraction collector with an electronic variable timer, which permitted the reproducible collection of sequential, precisely timed specimens of urine from each kidney. (The apparatus was designed by Dr F. Chinard and Mr J. Armant of Johns Hopkins Medical School, Baltimore, and built by Mr Armant.)

When the urine flow was adequate, the left renal artery was exposed, a curved No. 22 or No. 23 needle was introduced through the wall of the aorta near the origin of the renal artery and advanced into the latter approximately 1 cm. The urine fraction collector was started, and after two or three urine samples had been obtained, 1 ml. of solution, containing one or more isotopes together with 20–30 mg of creatinine, was injected rapidly (1–2 sec). The injected solution was made essentially isosmolal with blood by the addition of NaCl, and the pH was adjusted to between 7·40 and 7·50. When ^{45}Ca was used, the calcium concentration of the solution was made up to 0·1 mg/ml.

^{45}Ca (Catalogue no. Ca^{45}-P-3), ^{32}P(Catalogue no. P^{32}-P-1), ^{24}Na, and ^{42}K were obtained on allocation from the U.S. Atomic Energy Commission, Oak Ridge National Laboratory. ^{22}Na and ^{85}Sr were purchased from Nuclear Science & Engineering Corporation, Pittsburgh. The inulin-carboxylic acid-^{14}C (List No. 6795) was purchased from Abbott Laboratories Inc. with a specific activity of 0·26 mc/g. ^{28}Mg was obtained on allocation from the U.S. Atomic Energy Commission, Brookhaven National Laboratory, Upton, New York. Essentially carrier-free isotopes were used in all cases except ^{28}Mg. In the latter instance, approximately 10–20 mg Mg had to be used per injected dose, this being the maximum available specific activity. To make sure that all the phosphate-^{32}P was in ionic, rather than in complexed form, the radioactive phosphate solution was heated in a boiling water-bath for 20 min before use.

The injection was usually completed by the time approximately half of the first sample had been collected. In the calculation of transit time (see Table 1) and in the plotting of the excretion patterns, this delay was taken into account. Usually 30 urine samples, each collected over a period of 8–15 sec, were obtained from each ureter following the arterial injection.

In nearly all cases animals were used in two, and sometimes in three, successive experiments. At first a given experiment was repeated in two successive trials on the same animal. Later, animals were subjected to two different procedures in two successive experiments.

Creatinine. The urine samples were analysed for creatinine by a modification of the Jaffe method (Lauson, 1951).

Calcium. Analysis for calcium was carried out by a modification (Bronner, Harris, Maletskos & Benda, 1954) of the method of Salomon, Gabrio & Smith (1946). In general, urine and all plasma samples were wet-ashed with a 1:1 mixture of HNO_3-$HClO_4$ before analysis; only very dilute urine samples could be analysed directly by precipitation of the calcium with oxalate. Tissue samples were usually ashed in a muffle furnace before analysis was carried out (Bronner *et al.* 1954). When urine samples in individual collection tubes were to be analysed for calcium, the method of Kingsley & Robnett (1958) was employed, as it allowed analysis of samples containing 5–20 μg. The precision of the latter method was between 5 and 10% S.D., whereas a precision of better than 2% S.D. was achieved by the titrimetric analysis of the oxalate precipitate.

Sodium and potassium were determined by flame photometry, with lithium as an internal standard.

Phosphate analysis was carried out on the Technicon Autoanalyzer (Technicon Instruments Co.) by a method that is based on the phosphomolybdate colorimetric reaction originally described by Fiske & Subbarow (1925). It had previously been tested in our laboratory for conformance with two accepted manual methods.

Magnesium was determined by the titan yellow method of Andreasen (1957).

^{45}Ca or ^{14}C was determined on samples of 0·1 ml. urine or plasma that were diluted with 1·0 ml. H_2O containing three drops of a surface-active agent (1:30 Triton NE, Rohm and Haas Co.) and dried in cupped planchets under infra-red lamps. The samples were counted in automatic gas-flow counting equipment (Nuclear-Chicago) to accumulate no less than 2×1024 counts (background: $\sim$ 15 counts/min). In the early experiments duplicate samples were prepared, but when these agreed within the counting error their preparation in later runs was omitted. Samples were always counted along with standards that had been prepared by diluting an identical dose (pipetted out with the same syringe used for injecting into the animal) first with water, and then with either urine or plasma obtained from the experimental animal before injection. Tissue samples were analysed for ^{45}Ca content after chemical isolation of the calcium (Bronner, 1958).

Analysis for ^{85}Sr, ^{24}Na, ^{22}Na, ^{42}K, ^{28}Mg, and ^{32}P was done in the scintillation well-counter on 1·0 ml. samples of urine or plasma and compared with suitable aqueous standards (see above). As a single-channel pulse-height analyser was not available for the early experiments, they were designed so as to achieve separation by using isotopes of widely differing half-life. For example, if ^{85}Sr, ^{24}Na and ^{45}Ca were included in one test solution, the content of gamma emitter was determined as soon as practicable, and after nearly total decay of the ^{24}Na (7–10 half-lives). When correction was made for variations in counter efficiency and for the decay of ^{85}Sr, the difference in corrected counts was considered to represent the counts due to ^{24}Na. As ^{24}Na gives off an energetic β-ray, ^{45}Ca analyses were delayed until the ^{24}Na had decayed.

In later experiments a single-channel analyser was used along with the well-counter. This was essential for determining ^{28}Mg and ^{32}P activities, as ^{28}Mg is contaminated with ^{28}Al. Furthermore, in one experiment ^{32}P was injected into the same animal into which ^{28}Mg had been injected previously. This design necessitated counting the ^{28}Mg under conditions when the secondary photon emission due to the ^{32}P was suppressed, a condition met by the use of the voltage filter ('window') when counting at the spectral peak.

RESULTS

Figure 1 shows typical results obtained in an experiment in which a mixture of creatinine and ^{45}Ca was injected into the renal artery and the urine fractions analysed for ^{45}Ca and creatinine contents. In this and 31 similar experiments radiocalcium appeared in the urine earlier than the exogenous creatinine, a substance neither secreted nor reabsorbed by the tubular cells of the canine nephron (Smith, 1951).

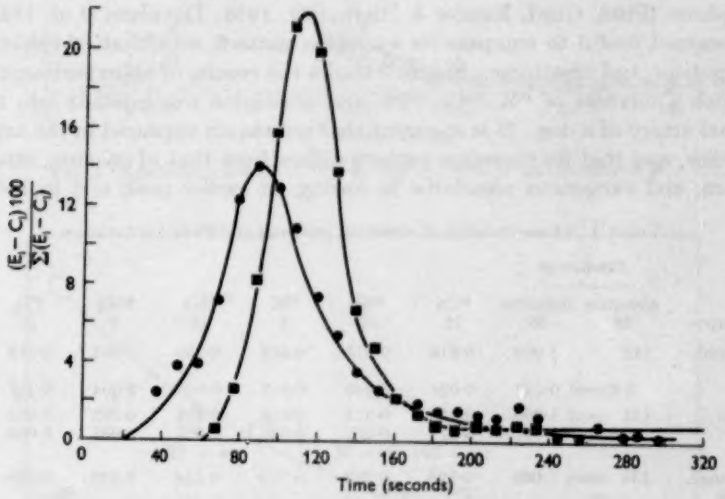

Fig. 1. Excretion patterns of ^{45}Ca and creatinine. 0·95 ml. of a solution containing 75 μc ^{45}Ca (●) and 30 mg creatinine (■) was injected into the left renal artery at zero time. Urine was collected separately from the left (experimental, E) and right (control, C) ureters in successive periods of 10 sec. Ordinate in this and following figures is as follows: the amount of the injected substance in the control urine (C_i) in each period (i) was subtracted from the amount on the experimental urine (E_i), and the difference ($E_i - C_i$) expressed as a percentage of the total amount $\left[\sum_{i=0}^{n}(E_i - C_i)\right]$ excreted by the experimental kidney. This represents the amount excreted in a single circulation (Chinard, 1955).

The absolute quantities of creatinine and ^{45}Ca that were excreted differed widely, as would be expected (Table 1). On the average 16·0% of the creatinine injected into the renal artery was recovered in the urine; this finding agrees with the common estimate that about one sixth of the renal plasma flow is filtered at the glomeruli. However, usually less than 1% of the injected ^{45}Ca was recovered in this manner (Table 1), reflecting the

high degree of calcium reabsorption known to occur in the kidney (Irving, 1957).

As strontium and calcium are closely related chemically, though their biological behaviour is not necessarily similar, it was of interest to compare their excretion patterns. When ^{85}Sr and ^{45}Ca were injected along with creatinine, the first two always preceded the third, though there was considerable overlapping between the ions (Figs. 2 and 3). The quantity of ^{85}Sr excreted always exceeded that of ^{45}Ca (Table 1).

Because potassium is known to be secreted in the distal part of the nephron (Pitts, Gurd, Kessler & Hierholzer, 1958; Davidson *et al.* 1958), it seemed useful to compare its excretion pattern with that of calcium, strontium and creatinine. Figure 3 shows the results of an experiment in which a mixture of ^{42}K, ^{45}Ca, ^{85}Sr, and creatinine was injected into the renal artery of a dog. It is apparent that potassium appeared in the urine earlier, and that its excretion pattern differs from that of calcium, strontium, and exogenous creatinine in having an earlier peak and in being

TABLE 1. Characteristics of excretion patterns of different substances

	Creatinine								
	Absolute	Relative	^{45}Ca	^{85}Sr	^{42}K	*Na	^{28}Mg	^{32}P	Inulin-^{14}C
No. of expts.	38	38	32	10	8	13	4	2	2
Mean transit time	142	1·000	0·816	0·815	0·865	0·739	0·963	0·915	0·965
S.E.	6·6 (sec)	0·047	0·028	0·040	0·047	0·005	0·004	0·015	0·005
Peak time	125 (sec)	1·000	0·803	0·812	0·518	0·658	0·993	0·955	0·980
S.E.	5·9	0·047	0·027 (n = 29)	0·032 (n = 9)	0·039	0·006 (n = 12)	0·003	0·005	0·010
50 % throughput time	134 (sec)	1·000	0·796	0·799	0·789	0·519	0·950	0·930	0·990
S.E.	6·2	0·047	0·022	0·037	0·035	0·113	0·031	0·020	0·000
Percent of dose	16·0	1·000	0·046	0·109	0·540	0·084	0·465	0·121	0·955
S.E.	0·7	0·047	0·010	0·022	0·098	0·002	0·059	0·022	0·025

*Na = either ^{22}Na or ^{24}Na

$$\text{Mean transit time} = \frac{\sum_{i=0}^{N}[(E_i - C_i)T]}{\sum_{i=0}^{N}(E_i - C_i)}$$

where $T = \frac{1}{2}(t_i + t_{i-1})$ and t_i = time in seconds when the collections E_i and C_i (see legend of Fig. 1) were completed.

Peak time is the time of the maximum of curves like those in Figs. 1–6.

50 % throughput time. A cumulative curve was drawn relating the amount already excreted to the time after injection; the time was then determined when 50 % of the total excretion had been reached

$$\left[\text{i.e. } 50\,\% \text{ of } \sum_{i=0}^{N}(E_i - C_i)\right].$$

Amount excreted = $\sum_{i=0}^{N}(E_i - C_i)$, expressed as a percentage of the dose.

For each experiment the values were expressed as a fraction of the value for creatinine. The figures in the table are the arithmetic means of these ratios, together with the standard errors of the means.

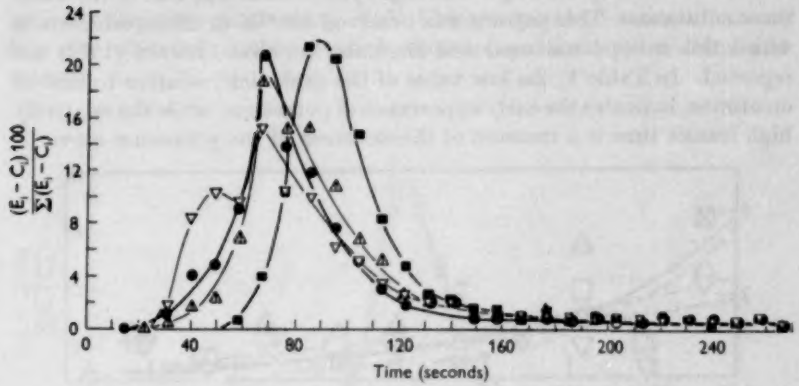

Fig. 2. Excretion patterns of ^{45}Ca ●, ^{85}Sr △, ^{24}Na ▽ and creatinine ■.

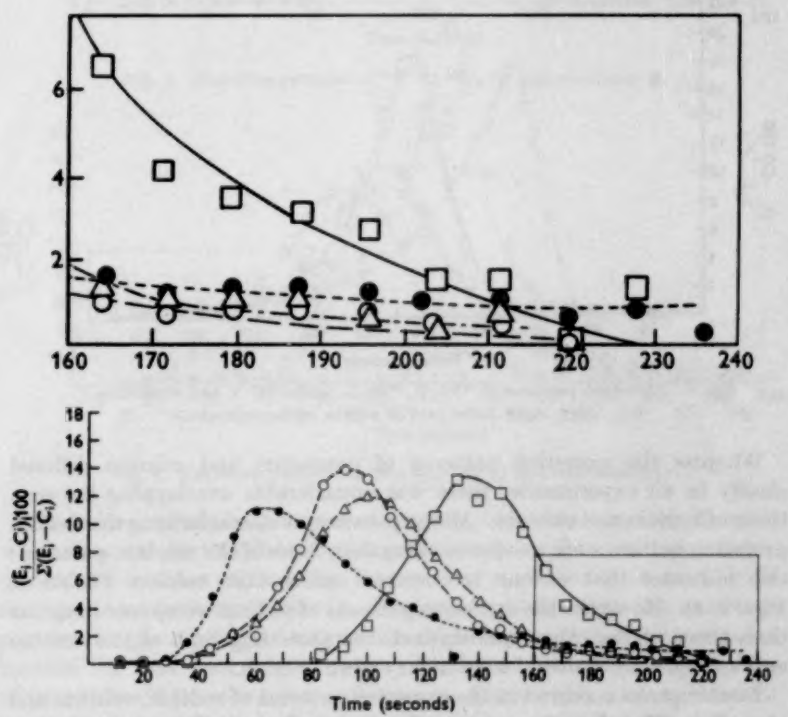

Fig. 3. Excretion patterns of ^{42}K ●, ^{45}Ca ○, ^{85}Sr △ and creatinine □. Inset, right-hand part of curves on larger scale.

skewed, excretion persisting for a longer period than was true of the other three substances. This pattern was observed for ^{42}K in all experiments in which this isotope was used and is similar to what Chinard (1957) has reported. In Table 1, the low value of the peak time, relative to that of creatinine, indicates the early appearance of potassium, while the relatively high transit time is a measure of the skewness of the potassium curve.

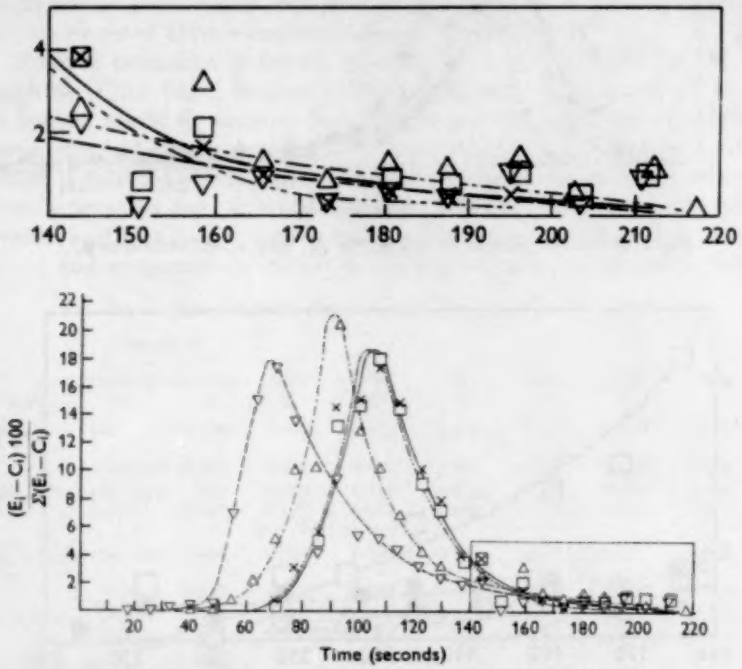

Fig. 4. Excretion patterns of ^{24}Na ▽, ^{85}Sr △, inulin-^{14}C × and creatinine □. Inset, right-hand part of curves on larger scale.

Whereas the excretion patterns of potassium and calcium differed clearly in all experiments, there was considerable overlapping between those of sodium and calcium. All the parameters characterizing the sodium excretion pattern were smaller in value than those of the calcium patterns; this indicates that sodium is excreted earlier than calcium (Table 1; Figs. 2, 4). However, the excretion patterns of sodium were more irregular than those of the other ions studied, the ascending limb of the sodium curve frequently showing a secondary peak (Fig. 2).

Double peaks occurred in the excretion patterns of sodium, calcium and strontium. Therefore the number of curves used to calculate the peak time

of these ions for Table 1 was smaller than the number of curves used to estimate the other parameters. F. P. Chinard (personal communication) has also observed doubly peaked excretion patterns for sodium and chloride.

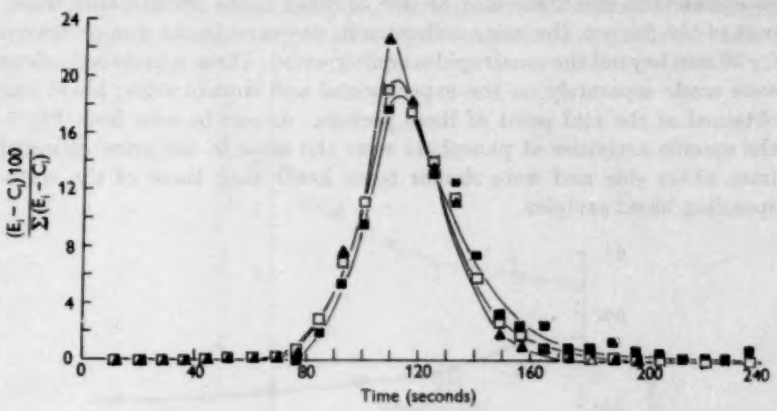

Fig. 5. Excretion patterns of ^{32}P ▲, ^{28}Mg □ and creatinine ■.

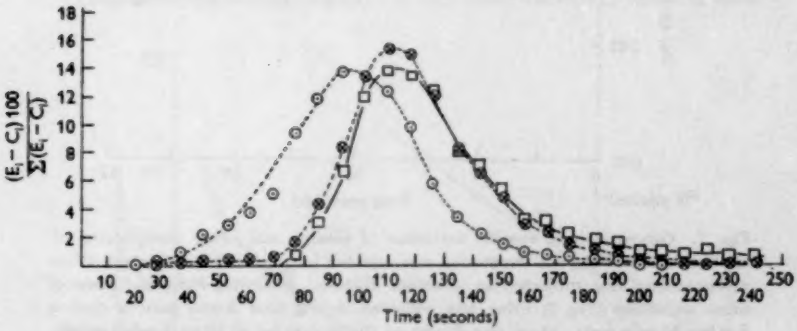

Fig. 6. Excretion patterns of ^{45}Ca ⊙, ^{28}Mg ⊗ and creatinine □.

The behaviour of phosphorus and magnesium is shown in Figs. 5 and 6, which demonstrate that the excretion patterns of phosphate and magnesium, in contrast to those of the other ions studied, were nearly identical with the creatinine pattern (Table 1). It seemed possible that the excretion patterns for phosphate and magnesium might resemble that for creatinine because the ions were taken up from the blood by tubule cells rapidly and then passed into the lumen only slowly. Conceivably some substances transported into the tubules at the same site might exhibit different

excretion patterns if there were large differences in their transtubular transport times. Alternatively, substances entering at different sites might show similar patterns.

To test the possibility that the similarity in excretion patterns between phosphate and creatinine may be due to delay in the transtubular transport of the former, the urine collection in one experiment was continued for 30 min beyond the usual rapid sampling period. Three-minute collections were made separately on the experimental and control sides; blood was obtained at the mid point of these periods. As can be seen from Fig. 7, the specific activities of phosphate were the same in the urine collected from either side and were similar to or lower than those of the corresponding blood samples.

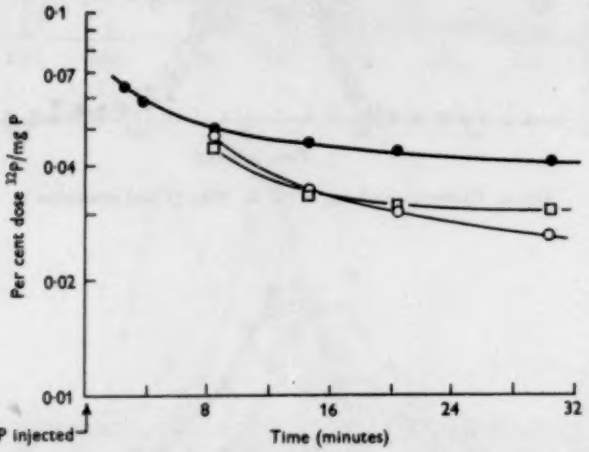

Fig. 7. Comparison of specific activities of plasma and urine phosphorus. A mixture of ^{32}P, ^{28}Mg and creatinine was injected into the left renal artery of an anaesthetized dog subjected to mannitol diuresis. Following the acute phase of urine collection (Fig. 5) urine was collected during four 3-min periods over a further 30-min span; blood was drawn at the mid point of these 3-min periods. Plasma ●; left ureter (experimental) ○; right ureter (control) □. Semi-log. scale.

In an analogous experiment the specific activity of magnesium in the plasma was compared with that in urine samples that had been collected for 2 min periods at 18 and 31 min following the injection of ^{28}Mg into the renal artery. As can be seen from Fig. 8, and as was also observed in a further experiment, the specific activities were essentially alike. The similarity of the specific activity values of the urines from the two kidneys and the plasma does not support the possibility of an appreciable, delayed influx of either phosphate or magnesium. On the other hand, a similar

comparison of the potassium in plasma with that in urine indicated that the experimental kidney excreted urine with a specific activity several times that of the control, which in turn equalled that of blood (Fig. 9).

A further indication of the difference between magnesium and calcium was brought out by tissue analyses. The experimental kidney of an animal given ^{28}Mg contained 11·2 % dose/m-equiv magnesium, whereas the control

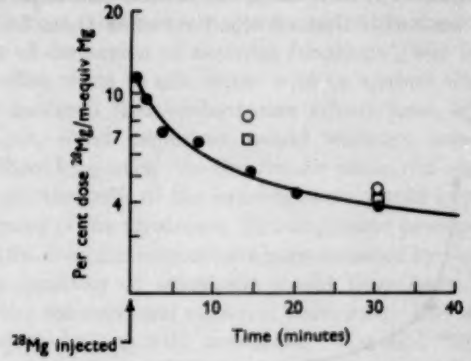

Fig. 8. Comparison of the specific activities of plasma and urine magnesium. Plasma ●; left ureter (experimental) □; right ureter (control) ○. Semi-log. scale.

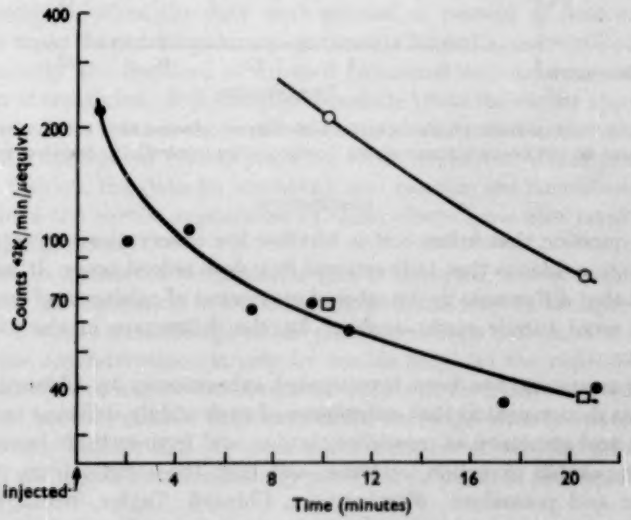

Fig. 9. Comparison of the specific activities of plasma and urine potassium. Plasma ●; left ureter (experimental) ○; right ureter (control). Semi-log. scale.

excretion patterns if there were large differences in their transtubular transport times. Alternatively, substances entering at different sites might show similar patterns.

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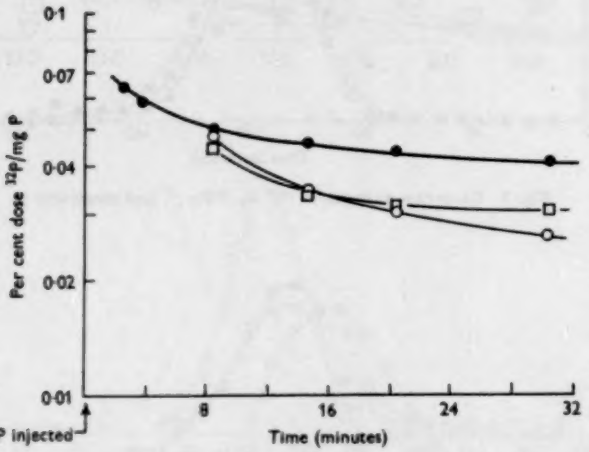

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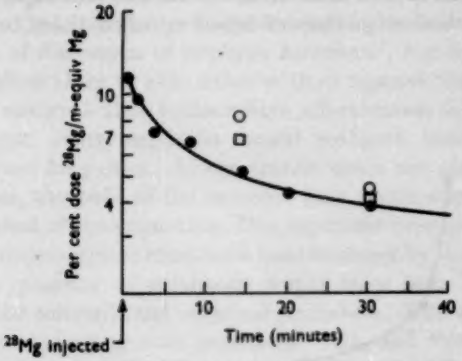

Fig. 8. Comparison of the specific activities of plasma and urine magnesium. Plasma ●; left ureter (experimental) □; right ureter (control) ○. Semi-log. scale.

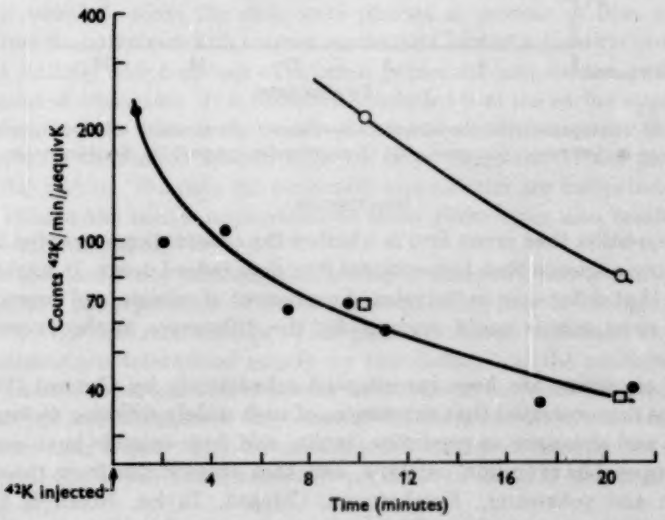

Fig. 9. Comparison of the specific activities of plasma and urine potassium. Plasma ●; left ureter (experimental) ○; right ureter (control). Semi-log. scale.

contained only 1·6% dose/m-equiv magnesium. Both kidneys had approximately the same magnesium content, averaging 9·7 μ-equiv Mg/g wet weight. In contrast, in six animals given ^{45}Ca the experimental kidneys had specific activities averaging only 1·6 times those of the controls (range: 1·0–2·1). The small quantity of calcium thus shown to be bound to kidney is not readily released into the urine, as a comparison of the specific activities of urine from the control and experimental sides with each other and with that of blood revealed them to be identical (Fig. 10).

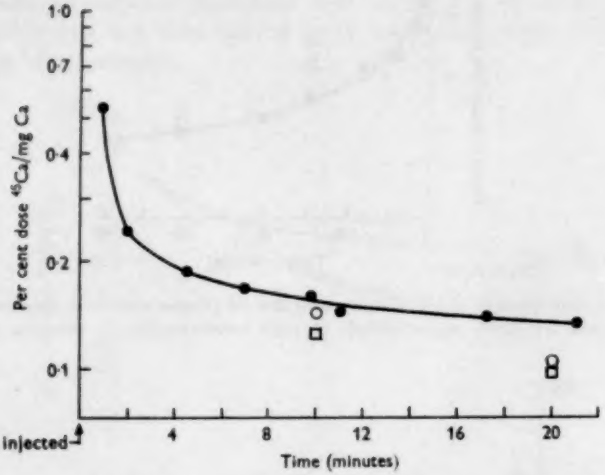

Fig. 10. Comparison of the specific activities of plasma and urine calcium. Plasma ●; left ureter (experimental) ○; right ureter (control) □. Semi-log. scale.

DISCUSSION

The question that arises first is whether the observations reported here constitute evidence that bidirectional flux does indeed occur. It might be argued that differences in the rates of movement of calcium and creatinine in the renal tubule could account for the differences in the excretion patterns.

This argument has been investigated exhaustively by Chinard (1955) who has demonstrated that substances of such widely differing molecular weight and structure as creatinine, inulin, and ferro-cyanide have nearly superimposable excretion patterns, and that these differ from those of sodium and potassium. Furthermore, Chinard, Taylor, Nolan & Enns (1957, 1959) showed that phlorrhizin treatment of a dog caused the previously different excretion patterns of glucose and creatinine to become

nearly superimposable. Similarly, Becker & Thompson (1959) caused the sulphate excretion pattern, which at endogenous sulphate plasma levels preceded the pattern for creatinine, to coincide with the latter following sulphate loading.

Another objection has been raised by Bradley & Wheeler (1958), who suggested that differences in peak excretion times might result from effective differences in nephron function. Chinard *et al.* (1959) have acknowledged the possibility that 'the delayed appearance of glucose in the urine could be the result of dispersion of nephron functions', but felt they had too few data to allow them to side either with or against this interpretation.

If it were assumed that reabsorptive effectiveness is proportional to nephron length, short nephrons would reabsorb lesser quantities of electrolytes than long ones. As the transit times are also shorter in the short nephrons, the bulk of the excreted ions would appear in the urine sooner than most of the creatinine. This argument presupposes that some, albeit very little, creatinine must have been excreted by the short nephrons. Possibly this quantity of creatinine would have been too small to be detected by the conventional chemical procedure. But when ^{14}C-labelled inulin was injected along with creatinine, ^{24}Na, and ^{85}Sr, the inulin and creatinine curves were essentially congruent (Fig. 4 and Table 1). Furthermore, a larger fraction of sodium (0·47 % of dose) had been collected than was represented by the first sample of inulin (0·005 % of dose).

In addition, when the data were plotted as percent of dose excreted against time, the resulting curves confirmed Chinard's observations (1957) that initially the fractions of injected potassium and sodium exceed the amount of creatinine. It is therefore concluded that the earlier appearance of these ions in urine is the result of transtubular influx rather than of a relatively diminished reabsorption by short nephrons. When plotted in similar fashion, the data for strontium and calcium are inconclusive, but we believe the earlier appearance of these electrolytes also results from transtubular influx.

If the occurrence of transtubular flux is accepted, the question arises as to how the separation in ion excretion patterns may be brought about. In our view, the relationships of ion patterns to each other and to that of creatinine are determined largely by two factors: (*a*) the relationship of the quantity that has moved across the tubule to the total excreted in the urine, i.e. whether most or only some of the ion in the urine is derived from transtubular influx; and (*b*) anatomical location of tubular influx, i.e. whether the site of flux is relatively proximal or distal in the renal tubule (Fig. 11).

Conceptually the simplest situation is one where most of the ion in the urine derives from transtubular influx, the bulk of the filtered ion having

been resorbed and the unresorbed fraction constituting only a small portion of the quantity recovered in the urine. In this case the degree of separation between the creatinine pattern on the one hand, and the ion pattern on the other, is a function of the site of transtubular flux (upper graph, Fig. 11). If flux occurs at a site relatively far from the glomerulus, separation will be clear-cut (solid curves of upper graph, Fig. 11); if flux takes place relatively close to the glomerulus, the curves will tend to overlap (broken and heavy solid curves of upper graph, Fig. 11). Similarly, if two ions cross the tubule at contiguous sites, their patterns will overlap;

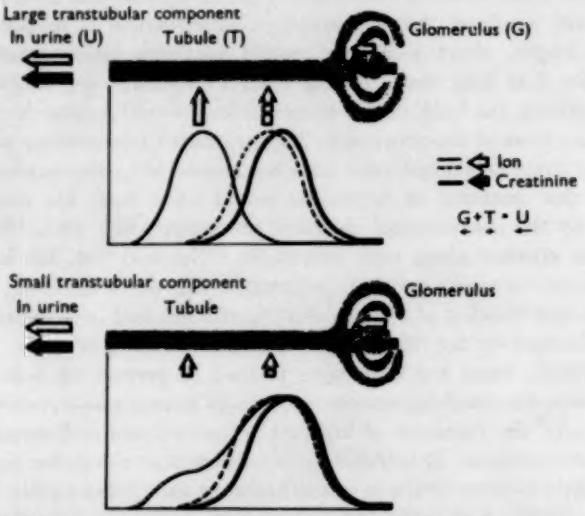

Fig. 11. Schematic representation of possible relationship between sites and quantities of transtubular flux, and excretion patterns; for details see text.

if ions cross the tubule at separated sites, e.g. one relatively far from and the other relatively close to the glomerulus, their patterns will be more distinct, the pattern of the latter being closer to and perhaps overlapping with that of creatinine.

On the other hand, if most of the ion in the urine derives from glomerular filtration, and if the contribution of transtubular influx to the quantity excreted is relatively small, most of the curve for the ion will coincide with that of creatinine, provided the site of ion flux is relatively close to the glomerulus (broken and heavy solid curves of lower graph, Fig. 11). If, under these conditions, ion flux occurs far from the glomerulus, a doubly peaked curve is likely to result (solid curves of lower graph, Fig. 11). Such a doubly peaked curve might also result if there were two sites of

ion flux. In that case, however, the second portion of the ion excretion curve would overlap the creatinine curve only partially. In the experiments reported here, doubly peaked curves have been observed occasionally (Fig. 2), but the second peak never coincided with that for creatinine.

Calcium. This ion appears to enter the tubule at a site relatively close to the glomerulus, perhaps in the proximal tubule. In accordance with the previous paragraph, one cannot assert whether most or only some of the calcium in the urine is derived from transtubular influx. In combined isotope and stop-flow experiments Samiy *et al.* (1960*a*) found that no measurable quantity of ^{45}Ca moved across the tubule into the urine at the distal site where reabsorption occurred. ^{45}Ca appeared in samples from the proximal tubule, peak excretion corresponding approximately with that for *p*-aminohippurate.

An autoradiogram prepared by Dr P. D. Saville showed maximum blackening due to ^{45}Ca in the convoluted tubule, near the glomerulus, and in the collecting tubules. ^{45}Ca concentration in the proximal nephron may mean that reabsorption of calcium takes place there. This interpretation concurs with our supposition and with that of Samiy *et al.* (1960*a*), namely that calcium flux in the dog kidney occurs close to the glomerulus, i.e. proximal of where potassium is secreted. It is tempting to speculate that regulation of calcium reabsorption in the kidney, which appears to be under parathyroid control (Buchanan, Kraintz & Talmage, 1959) is exerted at the site of transtubular calcium influx. The meaning in the autoradiogram of blackening due to ^{45}Ca in the collecting tubules awaits further work.

Strontium. Because the excretion patterns of calcium and strontium overlap and are similar, it is likely that both ions are handled in a similar fashion. However, the available evidence is also compatible with the possibility that influx is relatively small for one ion and relatively large for the other, the sites of flux being close to the glomerulus for both. On the average, 2·4 times more ^{85}Sr than ^{45}Ca was excreted (Table 1). This finding in dogs agrees with earlier reports for rats (Bauer, Carlsson & Lindquist, 1955), rabbits (MacDonald, Noyes & Lorick, 1957), and man (Spencer, Laszlo & Brothers, 1957; Spencer, Li, Samachson & Laszlo, 1960) and a recent report on clearances in dogs (Della Rosa, cited by Hursh, Lovaas, Piccirilli & Putnam, 1960).

Sodium. It would appear from the data reported here that sodium flux occurs relatively close to the glomerulus, though perhaps more distally than does calcium flux. The same reservation that applies to the calcium–strontium patterns applies also to the sodium–calcium patterns. Because doubly-peaked sodium excretion patterns have occurred, more than one site of sodium flux may exist. As Oken *et al.* (1959) have reported a rapid

bidirectional flux of sodium in the proximal tubule of *Necturus*, the larger peak may represent proximal and the smaller peak more distal influx.

Potassium. Flux of potassium occurs further away from the glomerulus than that of any other ion examined in these experiments. This observation accords with observations by Chinard (1957) and with what is known about potassium secretion from stop-flow experiments (Pitts *et al.* 1958). A significant feature of the potassium curve is its skewness, which, on the basis of the comparison of the specific activities of blood and urine (Fig. 9), can be attributed to rapid uptake and release of potassium by the tubular cells (Morel, 1955; Black, Davies, Emery & Wade, 1956; Chinard, 1957).

Phosphate. The occurrence of a small transtubular flux at a site near the glomerulus could result in the kind of curve shown in Fig. 5, corresponding to the pattern indicated by the broken line in the lower graph, Fig. 11. This interpretation is supported by the observations of Rees, Franklin, August, Small, Kendall, Merrill & Gibson (1959), who studied phosphate flux by the stop-flow technique and interpreted their findings as indicating that the flux of this ion occurs principally in the 'proximal tubular (PAH secreting) segment of the nephron'. Their results also demonstrate that the transtubular flux of phosphate into the luminal fluid is very small. In a very recent paper, Taugner, Egidy, Iravani & Taugner (1960) reported that following an intravenous injection of orthophosphate-^{32}P the isotope became located primarily in the proximal portion of the convoluted tubule of cat kidney. This they interpreted as the site of principal reabsorption of this electrolyte.

Carrasquer & Brodsky (1959) recovered in the urine a greater fraction of injected phosphate than of injected creatinine. This they interpreted to mean that phosphorus secretion can occur in the dog under special conditions of phosphate loading. Inasmuch as the work reported by Rees *et al.* (1959) and by us indicates a small flux, it seems likely that the net secretion of phosphate observed by Carrasquer & Brodsky (1959) was a function of the transient, but large, load of phosphate presented to the kidney.

Magnesium. The excretion patterns shown in Figs. 5 and 6 may have resulted from a small tubular influx which, in accordance with what has been discussed above, might occur near the glomerulus (lower graph, Fig. 11). The existence of transtubular influx of magnesium has also been reported on the basis of stop-flow experiments (Ginn, Smith, Hammarsten & Snyder, 1959; Wesson & Lauler, 1959; Murdaugh & Robinson, 1960; Samiy *et al.* 1960*a*). The findings of the last-named group of authors imply, as is also suggested by our interpretation, that the site of magnesium influx may be close to or identical with that for calcium.

The excretion patterns of magnesium and phosphate differed far more

from those of the other ions than from that of creatinine (Table 1). Indeed it is uncertain whether under our conditions the differences between the patterns of magnesium, phosphate and creatinine were significant. We have chosen to interpret our findings as compatible with small transtubular flux of these electrolytes. Another possibility needs to be considered, however. Transtubular transport may have been delayed sufficiently for the excretion patterns of these electrolytes to coincide with the creatinine pattern. Two types of observations bear on this possibility: measurements of the specific activities of the urines from the two kidneys and of the plasma obtained simultaneously; and analyses of the specific activity of the kidney itself.

If influx of a substance occurs beyond the time required for completion of the creatinine pattern, the excretion pattern for such a substance should extend beyond that of creatinine, as reported for *p*-aminohippurate (Chinard, 1956) and as observed for potassium (Fig. 3). Furthermore, for some measurable time after the creatinine has been excreted the specific activity of the urine on the injected side should remain significantly higher than on the control side or in the peripheral circulation, as in Fig. 9. As can be seen from Figs. 5 and 8 neither of these expectations was met, i.e. the excretion patterns of phosphate and magnesium did not extend appreciably beyond that of creatinine, and for both phosphate and magnesium the specific activities of the urines of the two kidneys neither differed from each other nor from the specific activity of the blood. Consequently an appreciable, but delayed, influx of either electrolyte seems unlikely.

We see no way of reconciling this conclusion with the report of Nicholson & Shepherd (1959), who studied the effect of tubular poisons on phosphate excretion in dogs and concluded that this ion is secreted actively by the distal tubule. Conceivably, as was implied by Carrasquer & Brodsky (1959), the mammalian kidney can, but does not normally, secrete phosphate. Moreover, in recent publications Samiy, Hirsch, Ramsay, Giordano & Merrill (1960*b*) and also Pullman, Lavender, Aho & Rasmussen (1960) reported that they were unable to find any evidence of tubular secretion of phosphate in dogs.

The ^{28}Mg content of the experimental kidney was ten times that of the control kidney one half hour after renal arterial injection of the isotope. Thus, a small influx of magnesium is not a result of poor cellular uptake of this ion. Rather, in contrast to potassium (Chinard, 1957; Morel & Guinnebault, 1958), it appears that the release of the large intracellular pool of magnesium into the urine is very slow.

The intracellular contents of calcium are small. The findings suggest that there is rapid uptake and release of this ion into the urine. It should

be noted, however, that a small amount of the injected ^{45}Ca remained in the kidney for at least 30 min. It seems likely, therefore, that some of the calcium entered a second compartment which turns over more slowly (Bronner, 1958).

SUMMARY

1. Creatinine and labelled electrolytes (^{45}Ca, ^{85}Sr, ^{28}Mg, ^{32}P, ^{42}K, ^{24}Na, ^{22}Na) were injected simultaneously into one renal artery of anaesthetized dogs subjected to mannitol diuresis and the patterns according to which these substances appear in the urine were determined.

2. Potassium, sodium, calcium and strontium preceded creatinine in the order named. It is concluded that a portion of these ions must have reached the urine by a transtubular influx.

3. Excretion patterns for magnesium and phosphorus were so similar to those for creatinine that only a very small proportion of these electrolytes in the urine is likely to have been derived from transtubular influx.

4. On the basis of these findings a scheme is proposed for relating excretion patterns to sites of transtubular influx.

5. Of the ions studied potassium was unique in that its uptake by kidney tissue was large and its turnover into urine rapid and continuing. Magnesium entered the kidney rapidly, but release into the urine was negligible. Calcium entered the tissues quickly and its turnover into urine was rapid, but continued influx, as seen with potassium, did not occur.

Our thanks to Dr E. Lovell Becker who kindly helped in carrying out some of the experimental work. Mrs Mary F. Dallman rendered skilful technical assistance. We also wish to thank Becton and Dickinson Co., Rutherford, New Jersey, and Rohm and Haas Co., Philadelphia, for gifts of some supplies.

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TEMPERATURE REGULATION OF SOME ANTARCTIC PENGUINS

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Penguins are widely distributed over the southern hemisphere from the Galapagos Islands (4° S) to the southern shore of the Weddell Sea (78° S). Two species predominate on the Antarctic continent, the Adélie penguin (*Pygoscelis adeliae*) and the Emperor penguin (*Apdenodytes forsteri*). Adélies breed in spring and migrate northwards in winter (Sladen, 1958), while Emperors, after breeding in winter, disperse in the Antarctic pack (Stonehouse, 1953).

Both species fast for long periods during the breeding season, the Adélie for over 40 days (Sladen, 1958) and the Emperor up to 120 days (J. N. Norman, unpublished). The birds are subject throughout their life to extremes of air temperature, from 10 to −60° C, and high winds, and a profound alteration in the seasonal light–darkness pattern of their environment. The very large number of penguins in the Antarctic points to a successful adaptation to the harsh environment. Our studies were made to elucidate the kind of adaptation that penguins have developed.

All measurements were made during the Austral summer of 1958–59 during the period of continuous daylight (Sladen & Goldsmith, 1960) in the Ross Sea area of Antarctica.

METHODS

Subjects. The subjects were three Emperor chicks and eighteen Adélie adults and chicks. The experiments were carried out either on board ship (U.S.S. Staten Island) with the penguins confined on deck in a small pen, or at one of three rookeries, Coulman Island Emperor rookery, or the Adélie rookeries at Cape Royds and Cape Hallet (Table 1 and Fig. 1).

Temperature measurements. Body-temperature measurements were made with a thermistor introduced into the stomach of the bird by a tube and rod introducer (Fig. 2), and connected to a resistance bridge, the accuracy of which allowed readings to be made correct to 0·1° C. In some experiments the thermistor was sheathed in thin rubber. This indwelling thermistor made it possible to take records continuously for 48 hr without undue disturbance to the bird. Foot-skin temperature was measured by a thermistor attached to the dorsum of the tarsus by adhesive tape.

TABLE 1. Species, age and condition of subjects together with the site where the experiments were carried out

No.	Species	Condition	Age	Site of observation	Nature of observation
1	Emperor	Chick in down	5 months	Coulman Island 73° 25′ S 169° 50′ E	Body temperature
2	Emperor	Chick in down through moult	5 months	U.S.S. Staten Island	Diurnal temperature changes Body and foot temperature changes in response to variations in environment
6	Adélie	Nestling in down	48 hr–15 days	Cape Royds 77° 25′ S 170° 55′ E	Body temperature changes in response to exposure
1	Adélie	Chick in down	6 weeks	Cape Hallett 72° 25′ S 170° 55′ E	Body temperature changes in response to immersion
3	Adélie	Adults	Over 1 year	U.S.S. Staten Island	
2	Adélie	Yearling moulting	14 months	U.S.S. Staten Island	Body temperature
6	Adélie	Adults	Over 1 year	U.S.S. Staten Island	

Variations in environmental temperature were produced by placing the bird either in a deep-freezer or in a heated room on the ship, which enabled us to vary the temperature from +26 to −44° C. Birds on board ship were confined for the periods of the experiments to a small pen on deck, separated from the other captive birds by a sheet of canvas. The birds were not fed during an experiment.

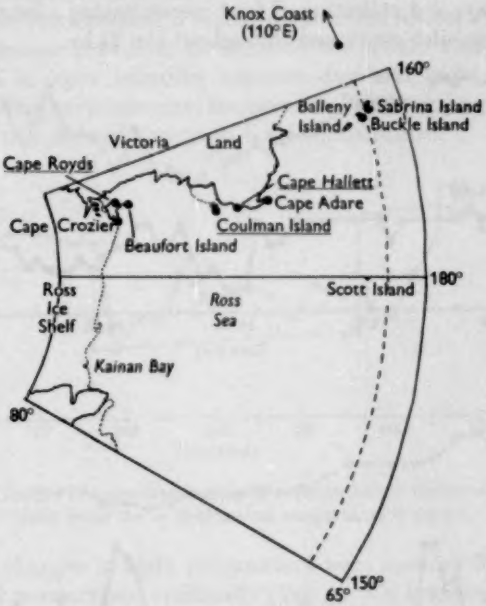

Fig. 1. Map of the Ross Sea area of Antarctica. ●, penguin rookeries; the names underlined were the sites of the experiments.

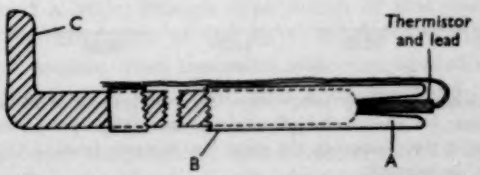

Fig. 2. The rod and tube introducer. The thermistor is held in the malleable jaws (A) of the tube (B) and the whole instrument is then gently pushed down the œsophagus to a pre-arranged depth (14 in. (35·6 cm) in Emperors, 12 in. (30·5 cm) in Adélies). Depressing the handle of the rod (C) frees the thermistor. The instrument is then removed.

RESULTS

Emperor chicks. At the Coulman Island Emperor rookery no obvious diurnal rhythm of activity was noted during one period of 48 hr of interrupted observation. Sleeping penguins were seen throughout the 24 hr of the day, and collecting of food, necessitating a long journey over ice to the water, also continued throughout the 24 hr.

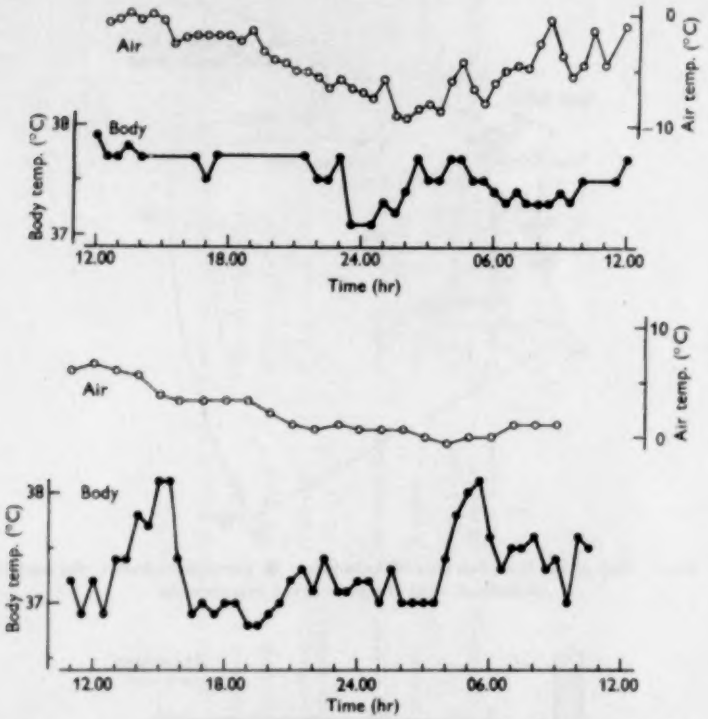

Fig. 3. Recordings of body temperature for two periods of 24 hr. The upper curves represent the diurnal changes of a 5-months-old Emperor chick; the lower curves represent the changes in the same bird 6 weeks later. In this and other figures 'body' = stomach.

On board ship, where the birds were on deck, 24-hr daylight conditions persisted throughout the experimental period. Noisy activity, especially from helicopters, continued most of the time. The birds showed no clear sleep–wakefulness pattern, sleeping during the lulls in man-made activity.

Figure 3 shows the result of two 24-hr periods of continuous temperature recording on the same bird. The upper curves represent an experiment

performed when the bird was 5 months old. It shows that the body temperature began to fall at about 22.00 hr and reached a minimum at midnight, 0·6° C lower than at midday, the environmental temperature having fallen in the preceding 6 hr by 5·3° C. The diurnal variation in body temperature seemed to follow changes in environmental temperature.

The lower curves represent a repeat experiment 6 weeks later. Though daylight conditions prevailed throughout the 24 hr, there was now a differentiation in light intensity between day and night. In spite of continually falling environmental temperature, there appeared to be some evidence of a true diurnal change in body temperature.

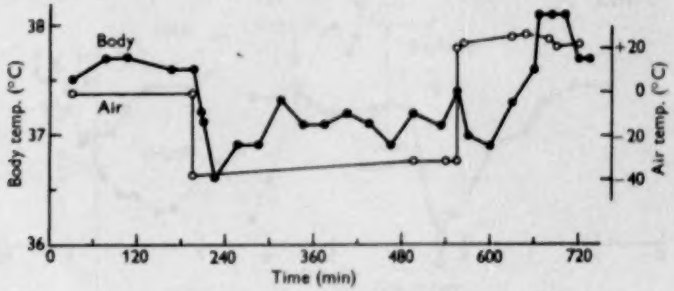

Fig. 4. Changes in body temperature of a 6-months-old Emperor penguin chick when the environmental temperature is varied.

Quite large changes in body temperature were induced by altering the environmental temperature artificially (Fig. 4). On lowering the ambient temperature from 0 to −40° C, the body temperature fell by 1 to 36·5° C; body temperature rose to 38° C when the ambient temperature was raised to 20° C. During the period of cooling the chick remained quite still and shivered a little, though observation of this phenomenon was hampered by the movement of the ship. In the hot environment the bird was at first restless, then lay on its abdomen, spreading its flippers, and panted. Respiration rate rose from a resting level of 5–6/min to 20/min. In one case respiration rose to 50/min.

Results of foot-skin temperature measurements showed that the surface temperature varied considerably with changes in environmental temperature (Fig. 5) but always remained well above zero in the cold environment. In both feet there was evidence of the hunting phenomenon of cold vasodilatation.

Adélie adults. The normal body temperature of the adult Adélie penguin at rest varied in our experiments from a mean of 37·7 (Fig. 6) to 40·2° C, the higher temperatures being recorded in two moulting yearlings (Table 1).

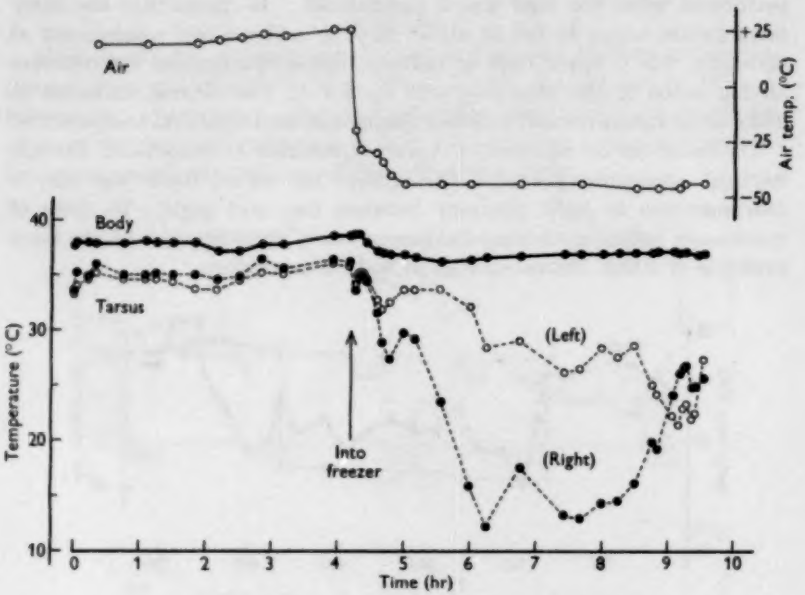

Fig. 5. Simultaneous measurement of foot and body temperatures of an Emperor chick in varying environmental conditions.

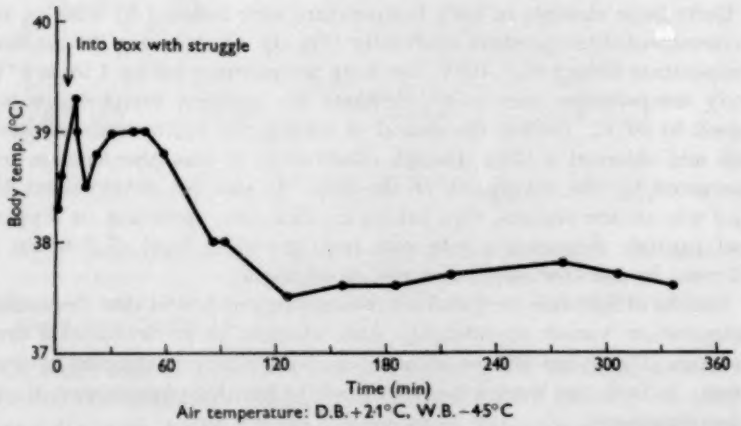

Fig. 6. Body temperature of an Adélie penguin adult, showing the length of time required for this to settle after the introduction of the thermistor and a struggle to replace the bird in an experimental box. Normal body temperatures were calculated from the mean after the initial fall had occurred (mean body temperature, 37·7° C).

All these results are the mean of at least 6-hourly or ½-hourly consecutive readings. The body temperature of these birds is raised rapidly by a struggle, often taking some hours to return to a steady level (Fig. 6), though the insertion of the thermistor, once experience had been gained, did not produce a great rise. This elevation of temperature can be brought about by trying to take a cloacal temperature; in one experiment a rise of 2° C was caused by repeatedly taking the cloacal temperature. In ten readings the cloacal temperature was 1·6° C lower than the stomach temperature taken simultaneously. One bird developed a pyrexia of 44·3° C 24 hr after the injection of an aqueous suspension of fungal spores into one hepatic air sac.

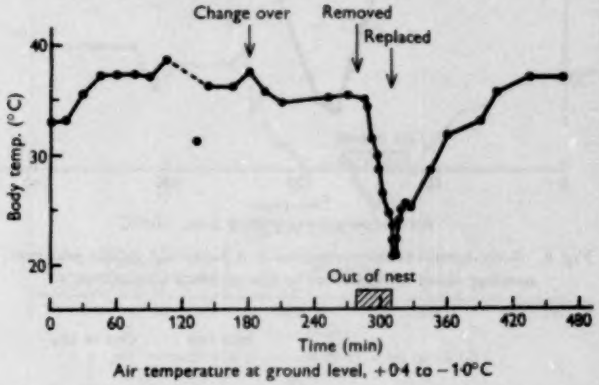

Fig. 7. Body-temperature variations in a 48-hr-old Adélie penguin nestling chick on exposure to the ambient conditions.

Adélie nestlings and chicks. The results of experiments on nestling chicks at the rookery are shown in Figs. 7, 8 and 9. Body-temperature measurements were first made in the nest, to obtain a normal resting value; the chick was then removed from the nest and exposed in the shade to the ambient temperature; finally it was replaced, to observe the rate of recovery. The body temperature of all nestlings dropped when they were exposed. All the chicks remained active, attempting to seek shelter. Neither the 48-hr-old chick (Fig. 7) nor the 5-day-old chick (Fig. 8) showed any signs of shivering, though the fall in body temperature was considerably less in the 5-day-old chick than in the other. The 10–15-day-old chick shivered vigorously as soon as it was exposed. All the chicks survived. Figure 10 shows the results from a chick, in down, taken from the crèche and aged about 6 weeks, which was placed in a water-bath at 0° C. The panting observed during the first part of the experiment may well be due to overheating as a result of the intense solar radiation which

is a feature of the Antarctic. Violent shivering started soon after the body temperature began to fall and continued long after the chick was removed from the water-bath. No fall in temperature was seen in adults similarly immersed.

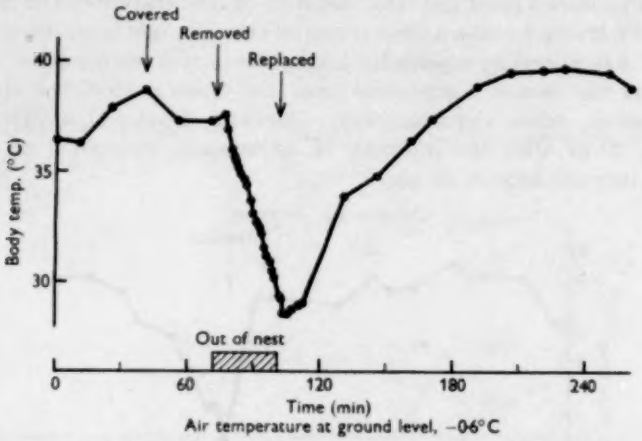

Fig. 8. Body-temperature variations in a 5-day-old Adélie penguin nestling chick on exposure to the ambient conditions.

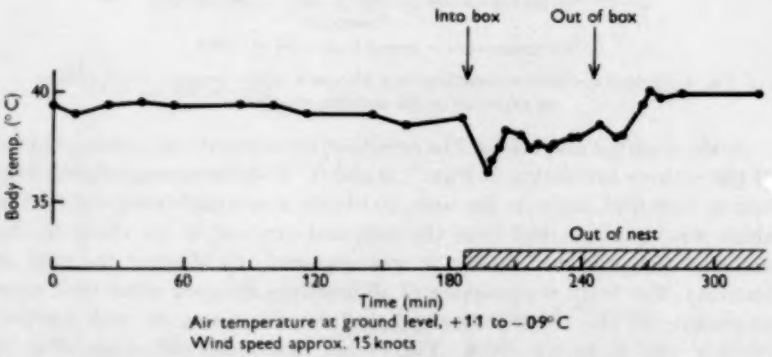

Fig. 9. Body temperature variations in a 10–15-day-old Adélie penguin nestling chick on exposure to the ambient conditions.

DISCUSSION

Previous investigators of penguin body temperature (Eklund, 1942; Prévost & Bourlière, 1957) have used readings from a clinical thermometer in the cloaca, but our results indicate the importance of using a technique

by which a continuous reading can be obtained. The rapid rise of body temperature due to struggling, and the slow return to normal, might well give grossly erroneous results if single readings only are obtained. This rapid rise in body temperature, which is similar to that found in Northern Fur Seals (*Callorhinus ursinus*) (Bartholomew & Wilke, 1956), is due to the rather poor development of a mechanism for heat loss in an animal adapted to the cold. Further evidence of this is the panting chick (Fig. 10), overheated at an ambient air temperature of 0° C by the intense solar radiation (L. G. C. Pugh, unpublished).

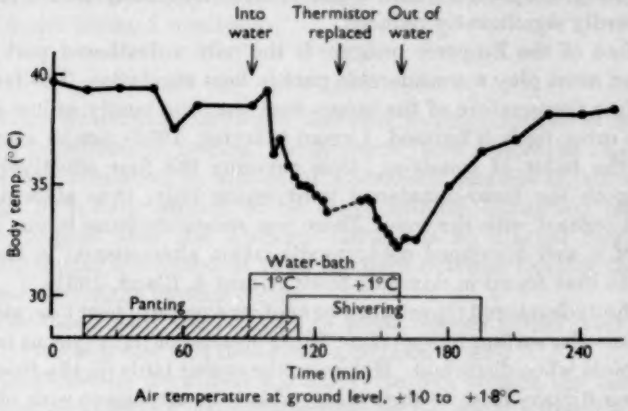

Fig. 10. Body temperature variations in a 6-week-old Adélie penguin chick on immerson in a water-bath.

Our results of body temperature measurement on the Emperor chicks showed an almost total absence of diurnal rhythm (Fig. 3), whereas 1 month later there is some evidence of a diurnal change. Irving (1955), however, showed a decline of up to 4° C at night, which he attributed to differences in level of activity—our birds were inactive throughout the experimental period. Neither chicks nor adult birds in the rookery showed any clear diurnal variation in activity, nor was there any easily discernible difference in light intensity between day and night; however, in the last 24 hr experiment the night sun was very low and a clear distinction between night and day existed. Armstrong (1954), in a review of the behaviour of birds in continuous daylight, concluded that light intensity is one of the factors that govern activity. Karplus (1952) came to the conclusion that the sleep–wakefulness pattern in birds was also governed by light intensity. Emperors, according to J. N. Norman (unpublished), show no obvious diurnal rhythm in activity during the period of total

darkness, and he has observed some sleeping birds throughout the 24 hr during continuous daylight. It is possible, therefore, that as the season progresses and the light–darkness pattern becomes more pronounced, so the diurnal activity and body-temperature rhythms are established, to be lost again during the polar winter.

The drop in body temperature in response to a decrease in ambient temperature has considerable survival value. The relatively labile body temperature control was demonstrated by Prévost & Bourlière (1957), who showed that Emperors in huddles during the incubating period had a lower body temperature than solitary birds; their daily weight loss was consequently significantly reduced.

The foot of the Emperor penguin is the only unfeathered part of the body and must play a considerable part in heat regulation. The fact that the surface temperature of the tarsus does not drop nearly as low as that in some other birds (Chatfield, Lyman & Irving, 1953) can be accounted for by the habit of squatting, thus covering the foot effectively, and standing on the tarso-metatarsal joint region only, thus allowing only minimal contact with the snow. There was strong evidence in our experiments of a well-developed cold vasodilatation phenomenon in the foot, similar to that found in domestic fowls (Grant & Bland, 1931).

The thinly-feathered flipper must be another avenue of heat loss; attempts to measure the surface temperature failed because of the vigorous flapping by the birds when disturbed. However, the cooled birds (in the freezer) all kept their flippers close to their sides—this is in agreement with observations in the field—but heated birds tended to sprawl on the ground spreading their flippers. The main channel of heat loss appeared to be through the panting mechanism.

Thermoregulation in the chick of Adélie penguins develops rather slowly and may govern the stages of behavioural development described by Sladen (1958). For 7 days the chick is entirely covered by its parent. When isolated it rapidly loses heat. It increases in size and develops the ability to shiver, but does not desert the nest entirely until this is well developed. Sapin-Jaloustre (1955) has shown that nestlings very rapidly increase in weight without much growth in length during the first few days after hatching. Thus the ratio of weight to length increases from 6·6:1 at 3 days to 14·2:1 at 5–7 days. This leads to a much more favourable weight-to-surface-area ratio. The shivering mechanism appears to develop between 10 and 15 days after hatching. Sapin-Jaloustre demonstrated that an Adélie is unable to survive outside the nest until it is 10–12 days old, death occurring in younger birds at a cloacal temperature of between 18 and 9° C. This is in contrast to the skua (*Catharacta MacCormicki*), living on the periphery of penguin rookeries, which forages for food,

leaving the newly-hatched chicks uncovered. Survival in water is made possible by the replacement of the wettable down by impervious feathers at the second moult when the chick is about 6 weeks old. This is unlike many species of water fowl, in which it is a common sight to see newly-hatched chicks taking to the water.

The apparently rather poor adaptation of the penguin chicks to their harsh environment must be accounted for partly by the lack of terrestrial predators, making it unnecessary for them to seek shelter in the water. However, it does make them very vulnerable to unfavourable weather conditions—the normally high mortality rate amongst chicks can rise to 100% under blizzard conditions.

SUMMARY

1. Body temperature of two of the most common species of Antarctic penguin (*Apdenodytes forsteri*, the Emperor, and *Pygoscelis adeliae*, the Adélie) was measured by means of a stomach thermistor, allowing recordings over long periods to be made without disturbing the birds. Results suggest that the Emperor chick has no diurnal temperature variation during the period of 24-hr daylight but may develop this when a normal day–night pattern occurs.

2. The mean body temperature of adult Adélies varied from 37·7 to 40·2° C, although one bird developed a pyrexia of 44·3° C.

3. Adélie chicks developed the ability to live outside the nest about 15 days after hatching, but could only enter the water without a drop in body temperature after they had moulted their down.

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MEMBRANE POTENTIALS IN MAMMALIAN CEREBRAL TISSUES *IN VITRO*: DEPENDENCE ON IONIC ENVIRONMENT

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Slices of cerebral tissues from guinea-pigs or cats can be maintained in moist oxygen at the surface of salines in a condition which permits the measurement of resting membrane potentials (Li & McIlwain, 1957). This technique gives an opportunity of comparing the effects of substances on polarization and metabolic phenomena in the slices. The present investigation examines the effect of the following cations on the membrane potential: potassium, sodium, calcium, magnesium and ammonium. L- and D-glutamate were also examined in view of their effect on the tissue content of potassium salts.

METHODS

In most experiments the cerebral cortex of the guinea-pig was used; occasionally experiments were done with rat, human, calf or sheep slices. Human specimens were obtained at operation for non-malignant conditions by Mr V. Logue and Mr P. Schurr, to whom we are grateful. Calf and sheep specimens were excised after the animals had been anaesthetized and used for other experiments by Dr F. Bell, to whom also we are grateful. Guinea-pigs and rats were exsanguinated and the brain excised as quickly as possible. Slices were cut tangentially to the surface of the parietal cortex, with a strip of razor blading, using a glass guide which gave slices 0·3–0·35 mm thick; these were transferred immediately from the blade into the incubating medium in the slice chamber (see below). The time elapsing between exsanguination and the beginning of incubation was 2½–3 min. Usually the superficial slice only was used, and it was pre-incubated for 40 min before recording.

The normal bicarbonate glucose saline contained (mM): NaCl 124, KCl 5, KH_2PO_4 1·24, $MgSO_4$ 1·3, $CaCl_2$ 2·8, $NaHCO_3$ 26 and glucose 10, and was equilibrated with 5% CO_2 in O_2. Its pH was 7·2–7·4 at the beginning of experiments, and never exceeded 7·8 by the end. Other salines are described in terms of their difference from this normal saline.

Apparatus

The apparatus was basically that of Li & McIlwain (1957), but differed in the following ways. The slices rested on a nylon grid mounted on a Perspex ring. The slice chamber and the water jacket which maintained the temperature at 37–38° C were made of a single piece of glass. In the assembled apparatus, a Tempunit pump supplied water to the outer jacket in a closed circuit from the thermostatically controlled bath. The 95% O_2 + 5% CO_2 gas mixture was passed through a flowmeter and a sintered-glass bubbler immersed in the

warm bath. The O_2 content of the gas phase above the slice was determined as described below and found to be between 70 and 80 %.

The slice chamber was mounted through Perspex rods on a heavy metal base 90 × 30 cm, which had been the table of a milling machine; the base rested on a Dexion frame, which had wood and rubber pads between it and the floor. It carried a Prior micromanipulator, tilted backwards at 30° from the vertical. The recording micro-electrode fitted into a half cell of 3 % agar in 2·7 M-KCl, with an AgCl–Ag wire plugging into a miniature socket. This was connected to the grid of a cathode follower, the length of the lead between the micro-electrode and the grid being kept to a minimum, so as to reduce the contribution made by the lead to the input capacity.

The fine adjustment of the micromanipulator carried a pointer with which the depth of penetration could be measured on a calibrated scale; 1° was equivalent to advance of the tip by 0·3 μ, or to penetration of the slice to a vertical depth of 0·25 μ.

The micro-electrodes were glass micropipettes, made from Pyrex tubing of internal diameter 1–1·5 mm and wall thickness about 0·3 mm. They were drawn in a Palmer micro-electrode puller, after the design of Alexander & Nastuk (1953), to tips of $< 1\,\mu$. About 20 electrodes were made in one batch and filled with 2·7 M KCl solution under reduced pressure. Their tips and d.c. resistances (15–100 MΩ) were examined before use. It was found necessary for the shank of the electrode to be at least 1·5 cm long, and for the taper at its tip to be less than 1 in 10.

The cathode-follower valve was a paired acorn pentode RCA 954 with a grid current of $< 10^{-13}$ A; it was balanced by another cathode follower whose input was at earth potential. The amplifier used was a two-stage push-pull d.c. amplifier with negative feedback, designed by Dr J. F. Palmer & Mr G. L. Reid, to whom we are grateful for the circuit diagram. It was constructed by Mr J. Westhenry. The trace was displayed on a Cossor oscilloscope Type 1049, to which a camera was sometimes attached.

Procedure

The thermostat heater and water circulation were switched on while the electrodes were being made, and the saline was gassed with 5 % CO_2 in 95 % O_2. The medium (10 ml.) was put into the slice chamber which was closed and the gas passed over it at the rate of 100–125 ml./min. Two or three slices totalling 1·5 cm^2 in area and each weighing 40–80 mg were cut promptly, placed in the chamber, and left at 37° C for 40 min. This period of pre-incubation was chosen because reliable readings could not be obtained before 20–30 min.

Electrical observations were commenced by advancing the electrode by its micro-manipulator until it touched the surface of the solution above a slice; the amplifier was then balanced, and slight tip potentials and inequality between half cells balanced off; electrodes with large tip potentials were rejected. The electrode was then withdrawn from the solution and moved slightly to a new position above the slice. It was then advanced into the tissue with the fine adjustment of the micromanipulator, and deflexions on the oscilloscope lasting more than 100 msec were noted. A calibration signal was frequently put through the recording system and this gave an indication of a blocked electrode by its increased time constant, as well as a check on the recording system. In a typical experiment 20–25 slow penetrations through 0·36 mm, corresponding to a vertical depth of 0·26 mm in the slice, were made from randomly chosen parts of the slice; the solution was then changed or additions made to it, a further 20–25 readings recorded, and where relevant the original solution reintroduced through the syringe. Ten to twenty minutes elapsed after any change of solution before the readings were taken.

The readings quoted are the maximum negative potentials recorded in each penetration to a depth of 0·26 mm. If no potential was recorded in three successive penetrations, or there was evidence—like drift or a long time constant—of a damaged electrode, it was taken out and examined with a water immersion objective at a magnification of 1350. When normal

tissues and media had been used, absence of recordable potentials was usually due to a damaged electrode; when this was seen to be the case, the zero readings were not taken into account when appraising the tissue potential. If, however, the electrode appeared normal, the previous zero readings were regarded as a property of the tissue. In solutions which depolarized the tissue, a longer time was spent in obtaining a given number of readings, as the electrodes were examined frequently to ensure that such zero readings were due to cell depolarization and not damaged electrodes.

Chemical determinations

At a chosen stage during an experiment a slice was removed from the chamber by a bent wire which could be hooked on to a torsion balance. The slices were taken out rapidly, dipped for 3 sec in ice-cold 0·25 M sucrose, drained by repeated contact with a glass slide, and weighed. They were then treated in one of two ways.

(*a*) Homogenized in 3 % trichloroacetic acid at 0° C, left for 20 hr at room temperature and diluted: determinations of K^+ and Na^+ were carried out with an EEL flame photometer; chloride was estimated by an adaptation of Lowry's method (Cummins & McIlwain, 1961). These three ions were kindly estimated by Dr J. Cummins.

(*b*) For tissue phosphates slices were ground in 1·5 ml. of 10 % trichloroacetic acid in a 5 ml. test-tube homogenizer which could be centrifuged, and determinations carried out by a procedure based on that of McIlwain (1959). These estimations were done by Mr R. Woodman.

After incubation for 80–120 min in the normal saline described above, the slices contained (mean, S.D., and number of observations): potassium, $60·4 \pm 8·5$ (12); sodium, $102 \pm 4·6$ (6) and chloride, 45 ± 15 (6) μequiv/g tissue. Values for tissue constituents after incubation in modified salines are quoted in the corresponding Results section.

The tissues were found to absorb fluid during incubation, as occurs during most *in vitro* experiments (see Pappius & Elliott, 1956). On weighing slices after draining, and then drying them to constant weight (weighing after 2 and 3 hr at 100 °C) their water content after incubation for 1 hr in normal saline was found to be $83·3 \pm 1·3$ % (mean and S.D. for 5 experiments). The water content of the fresh tissue was 80·0 %, implying the uptake during incubation of fluid equivalent to 20 % of the fresh weight of the tissue. The following values for tissue constituents (mean and S.D. for 5 expts.) have been referred to the original fresh weight of tissue by using these data:

Phosphocreatinine, after incubation for 1 hr in normal saline, $1·67 \pm 0·30$ μmole/g; after an experiment involving about 100 penetrations with a micro-electrode, $1·63 \pm 0·37$ μmole/g.

Inorganic phosphate, after incubation, $2·90 \pm 0·42$; after penetration as above, $2·90 \pm 0·28$ μmole/g.

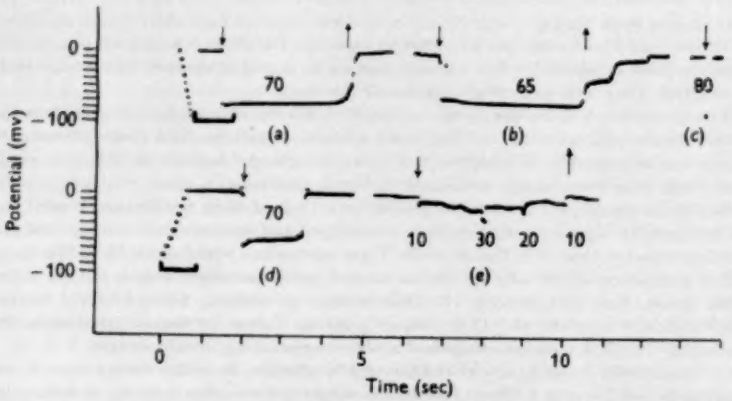
Oxygen and carbon dioxide tensions in gases were measured in the Scholander apparatus; 2 ml. of gas from the space above the slice was drawn into a syringe lubricated with petroleum jelly and sealed with mercury. Two estimations were done on each sample and a third if the first two differed by more than 1 %.

RESULTS

Resting potentials in normal glucose saline

On advancing the micro-electrode into the slice sudden changes in potential were recorded. Plate 1 is a photomicrograph of the first two slices of the guinea-pig brain showing the six layers of Brodmann in which cells were penetrated. Text-figure 1 shows the negative potential changes; examples include (*a*) a negative change of 72 mV, gradually being reduced before the electrode was withdrawn. In (*d*) the return is more rapid;

this is the change most frequently seen in normal tissue. The potential in (*b*) is gradually increasing. In (*c*) a sudden very short-lasting change is seen. This reading would be discounted, as would that of (*e*), which is presumed to be the result of a temporary blockage of the electrode tip, which could not however be seen on subsequent examination of the electrode.

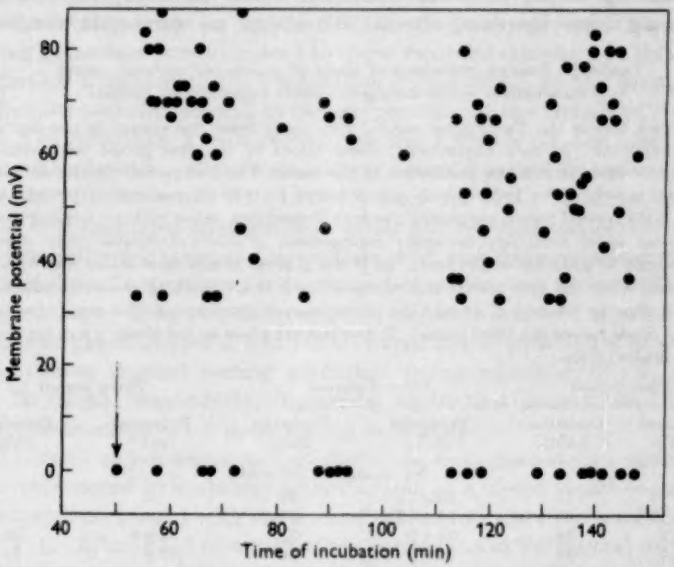


Text-fig. 1. Photographs of a number of penetrations of single cells of cerebral slices *in vitro*. Downward arrow indicates penetration, upward one indicates withdrawal. Horizontal bar, 1 sec. Numbers shown against records are potentials in mV; (*c*) touched up. For description see text.

Return of potential to its initial steady level was almost always less abrupt than the change on penetration. Change in potential on penetration, also, was less abrupt than that shown in Text-fig. 1 when the tissue was incubated under certain adverse conditions, for example, in media without added Ca or Mg salts. Resting potentials recorded under normal conditions could often be increased by minute adjustments of the micromanipulator by distances of less than 5μ, and this was always tried. Potential changes which did not return to the previous level spontaneously or on withdrawal of the electrode were regarded as having been modified by changes in the electrode, and are not included in results reported. All potentials so reported lasted at least 0·1 sec. When in a few cases their stability was examined over longer periods, they frequently maintained their maximum level for 10 min, some still being observable after 25 min.

The first part of an experiment in which the average resting potential was determined is shown in Text-fig. 2. This was a slice of human tissue, which was not observed to differ in the present experiments from guinea-

pig tissue. Its mean resting potential was 57 ± 10 mV (mean and S.D.) in 93 readings, the experiment lasting altogether for 4 hr, with 20 zero readings. Many potentials over 70 mV were recorded. In a series of 12 experiments with guinea-pig parietal cortex 236 readings gave a potential of 59 ± 12 mV (mean and S.D.).



Text-fig. 2. Resting membrane potentials from a human cerebral slice *in vitro* in oxygenated Krebs–Ringer bicarbonate glucose saline. Each point is a single maximum reading from a penetration 260 μ deep. Arrow indicates beginning of recording after pre-incubation. Note (*a*) the wide scatter of readings within one experiment under optimum conditions, (*b*) the many readings over 70 mV, (*c*) the number of penetrations with zero readings, (*d*) the ability of the tissue to maintain the polarization of some cells for 150 min. The intervals in recording are due to examination and replacement of damaged electrodes. The absence of values between zero and 30 mV is related to the criteria chosen for replacement of electrodes (see p. 264).

Cell size and position. The following comparison has been made, in the guinea-pig, between the size of the regions of negative potential, and the size of cells in stained sections of the same tissue. The distance travelled by electrodes while recording uninterrupted negative potentials on 50 occasions was $18 \pm 13 \mu$. The size of intersected portions of 50 cells in the slides from which Pl. 1 was prepared, when measured along 5 axes 1·5 mm apart and at an angle of 30° to the vertical (the micro-electrodes were inserted at this angle) was $12 \pm 4 \mu$. Shrinkage during histological pro-

cedures appears likely to help to make this value less than the distance of travel of the electrode. Both dimensions are markedly smaller than the average cell diameter, because an electrode or an intersecting line can only occasionally be expected to pass through the centre of a cell.

Dependence of the potentials recorded on the depth of the micro-electrode tip in the slice was examined in the three experiments with guinea-pig tissue, involving about 150 readings; no correlation was found.

TABLE 1. Resting potentials of slices of guinea-pig parietal cortex in bicarbonate saline during successive experimental periods

Each line of the Table gives results with tissue from one animal in one day's experiments. In each experiment, observations of the first period were commenced after 40 min pre-incubation of the tissue. The first period comprised the time, noted in the Table which was required to take 25 readings of potential. In A the second period comprised the next 25 readings, taken without altering the system apart from any necessary replacement of micro-electrodes (they were replaced as specified in the text). In B the glucose bicarbonate saline was withdrawn after the first period and replaced by a fresh batch of the same saline, and after an interval of 10 min the second period commenced. The same change was made before the third period. Potentials are given as the mean ± S.D. for the 25 observations.

First period		Second period		Third period	
Potential (mV)	Duration (min)	Potential (mV)	Duration (min)	Potential (mV)	Duration (min)
		A. No change in the saline			
52 ± 12	27	53 ± 11	40	—	—
51 ± 11	40	54 ± 12	37	—	—
52 ± 7	54	56 ± 9	39	54 ± 8	55
49 ± 7	18	53 ± 7	30	55 ± 7	14
		B. Saline changed between successive periods			
62 ± 9	43	61 ± 10	40	47 ± 10	50
55 ± 4	104	50 ± 9	85	52 ± 3	52
53 ± 14	40	52 ± 13	32	53 ± 10	28
61 ± 8	21	63 ± 8	25	62 ± 10	29

Correlation was sought also between the distance which the electrode travelled within a region of negative potential, and the magnitude of the potential. Again no correlation was found. Electrodes were found to be in regions of negative potential for an average distance of 26μ in each 100μ travelled (80 penetrations in 4 experiments). In 2 experiments with liver slices the electrodes were found to be in regions of negative potential for 56μ in each 100μ travelled (see Li & McIlwain, 1957).

Change of medium and excision of tissue. The trend of resting potentials in successive experimental periods was studied first without change of solution, and secondly with change of solution for one of the same composition. It can be seen from Table 1 that in general no significant changes in potential occurred in successive periods of either type of

experiment. The time required to take 25 readings of potential in each experimental period is recorded, as it was variable and could have conditioned the potentials observed. No correlation is, however, discernible in the findings in this respect in Table 1. (In earlier experiments, before the techniques described were fully established, the resting potentials measured averaged 35–40 mV, again without variation in successive experimental periods in which the salines were replaced. Values for resting potentials were increased to those recorded elsewhere in this paper by further attention to the preparation of the tissue, to the preparation of the micro-electrodes, and to the oxygenation of the chambers.)

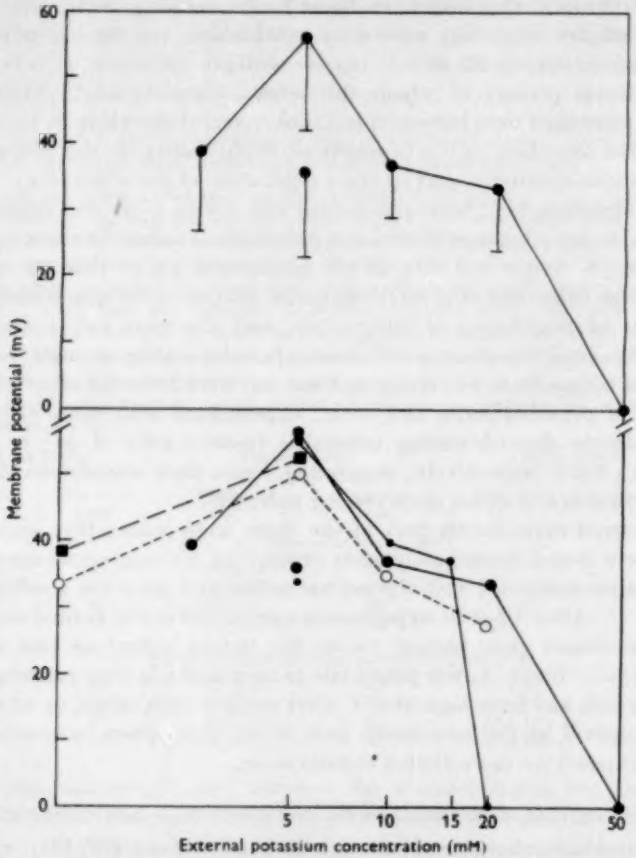
In 3 experiments, 2 with guinea-pigs and 1 with a rat, the brains were removed under ether anaesthesia and gave normal values for resting potentials: 60 ± 8, 51 ± 8 and 62 ± 12 mV (mean and S.D.). Human cerebral cortex was examined after excision under nitrous oxide anaesthesia after previous administration of thiopentone, and also gave values similar to those of normal experiments with tissues from laboratory animals (Table 1). Cerebral tissues from two sheep and one calf were removed after administration of pentobarbitone and ether; experiments with slices of each of these tissues showed resting potentials (mean $\pm$ S.D.) of 54 ± 6, 52 ± 4, and 70 ± 8 mV respectively, suggesting again that anaesthesia did not have a deleterious effect upon resting potentials.

In several experiments part of the brain from guinea-pigs, sheep and man were stored immediately after excision in a closed vessel containing filter paper moistened with the normal saline, and placed in a refrigerator at 2–4° C. After 1·5–5 hr experiments carried out in the normal way with such specimens gave normal values for resting potentials (see also Li & McIlwain, 1957). Lower potentials or no potentials were recorded from tissue which had been kept at 4° C after contact with saline, or which had been exposed in the laboratory (not in an atmosphere saturated with water vapour) for more than a minute or so.

Dependence of potentials on the concentration of potassium salts

The normal saline contained 5 mM-KCl and 1·2 mM-KH_2PO_4; the KCl was omitted and the phosphate replaced by the sodium salt in the experiments of the present section, and when necessary chosen amounts of concentrated KCl were added to give the potassium concentrations recorded. In 5 experiments potassium-free media were used. After incubation with the tissue, these were found to contain nearly 1 mM-K^+, derived presumably from the slice. No resting potentials could be recorded in these media; the slices from them were found to contain (μmole/g tissue, with S.D. and number of experiments) K, 44 ± 7 (6); Na, 117 ± 15 (6); and Cl, 48 ± 11 (4).

Resting membrane potentials observed in slices incubated in media with increasing potassium concentrations are shown in Text-fig. 3. Potentials with the normal external [K^+] of about 6·2 mM were greater



Text-fig. 3. Resting potentials in cerebral slices, and the potassium concentration (log scale) of incubating salines. Above: results from a single slice initially in 3 mM-K^+ obtained during successive additions of KCl, each circle giving the mean of 25 readings, with the S.D. represented by a vertical line. When the tissue had been depolarized for 100 min, it was returned to 6·2 mM-KCl and the lower circle at 6·2 mM-KCl shows the potentials recorded at that concentration in the cellular elements of the same slice which has repolarized. Below, mean resting potentials from several similar experiments (indicated by different symbols) with different external potassium concentrations. The lower readings at 6·2 mM-KCl are from tissue which had been returned to this concentration after depolarization with a higher concentration of KCl.

than those found in 4 or 10 mM. Potentials fell to zero with 20–50 mM-K^+. Tissues so depolarized could to some extent regain their potentials after being restored to the normal saline of 6·2 mM-K^+. This is noted in two experiments of Text-fig. 3, with tissues which had been depolarized for 100 and 120 min, respectively. That the restoration of resting potentials was not complete may have been due to the incubation *in vitro* having then lasted for 3 hr.

Sodium concentration and resting potentials

In 5 experiments, in which the NaCl of the normal saline was replaced by an equimolar concentration of choline chloride, and the $NaHCO_3$ by equimolar choline bicarbonate, no resting potentials could be observed. After 80 min incubation in three of these experiments, solid sodium chloride was added to bring the sodium concentration to normal. The media were then hypertonic (see below); no potentials were observed. Eight slices were analysed after incubation in media without added sodium salts, giving (μmole/g. tissue, $\pm$S.D.): K, 30 ± 6; and Na 14 ± 4. Three of the slices in media to which NaCl had subsequently been added, gave values: K, 36 ± 1; and Na, 67 ± 4 μmole/g. Thus the slices had regained some Na, but little K. That the choline did not affect the resting potential was shown in two experiments in which $NaHCO_3$ buffer was replaced by choline bicarbonate (26 mM) and normal potentials of 54 ± 12 mV (S.D.) and 58 ± 11 mV (S.D.) were recorded, while the slices contained 50 and 54 μmole/g of K and 60 and 62 μmole/g of Na, respectively.

By contrast to the findings in Na-free media, lowering the Na content of the medium from 150 to 26 mM resulted in no significant change in potential, nor did raising the Na to 300 mM (Table 2). The latter solution was markedly hypertonic.

Calcium and magnesium concentrations, and resting potentials

In a normal Krebs–Ringer bicarbonate saline with 2·8 mM-Ca^{2+}, resting potentials could be observed after 20–30 min incubation. In 5 experiments the calcium was omitted from the medium, and potentials could not be observed until 60–70 min, but then sometimes appeared; in 2 experiments values were 51 ± 12, and 53 ± 20 mV (mean and S.D.; $n = 25$ each). Subsequent addition of calcium to make the solution 2·8 mM after potentials had been observed in its absence, produced no further change.

In 3 experiments the tissue was incubated in normal glucose saline with 2·8 mM-Ca^{2+}, and when this was replaced by 0·56 mM-Ca^{2+} the resting potentials changed from 59 ± 14 to 56 ± 12 mV (mean and S.D.; $n = 75$). When in 4 experiments the initial saline contained 0·56 mM-Ca^{2+}, and the concentration was raised to 2·8 mM, the potentials changed from 56 ± 12

to 56·5 ± 11 mV (mean and S.D.; $n = 100$). In 4 experiments the Ca^{2+} concentration was raised from 2·8 to 14 mM in one step, the potentials changed from 54 ± 12 to 55 ± 14 mV (mean and S.D.; $n = 100$). None of these potential changes were significant.

Absence of magnesium from the external solution also caused a delay in repolarization of cells, and when after incubation of 60 or 70 min potentials appeared, they were slightly lower than in other experiments of this series, being in these experiments (mean and S.D.): 46 ± 14; 48 ± 15 (60 readings), and 52 ± 9 mV. In two experiments addition of $MgSO_4$ to make the final concentration 1·3 mM caused no significant change, the potentials initially being (mean and S.D.) 52 ± 9 and 45 ± 11 mV, and on addition of $MgSO_4$ to 1·3 mM 52 ± 10 and 50 ± 9 mV respectively.

TABLE 2. Sodium salts and resting potentials (mV) during successive periods

Experimental arrangement and recording of results as in Table 1*B*, with media of the Na content indicated. Media with 26 mM-Na^+ contained 124 mM-choline chloride. Additional Na in *B* was added as NaCl. Guinea-pig cerebral cortex (except in expt. *, which was with human cortex)

A

First period, normal saline	Second period, 26 mM-Na^+	Third period, normal saline
48 ± 10	42 ± 10	42 ± 8
53 ± 8	53 ± 12	45 ± 6
54* ± 10	59* ± 14	58* ± 7
49 ± 10	45 ± 8	43 ± 7

B

First period, normal saline	Second period, 225 mM-Na^+	Third period, 300 mM-Na^+	Fourth period, normal saline
60 ± 12	56 ± 9	44 ± 20	46 ± 15
57 ± 14	56 ± 13	32 ± 13	—
52 ± 8	49 ± 10	55 ± 12	62 ± 15
54 ± 10	—	54 ± 12	—
63 ± 9	60 ± 11	—	—
58 ± 13	—	59 ± 13	56 ± 13

In the second series of experiments concentrated $MgSO_4$ was added to a solution containing the normal 1·3 mM-$MgSO_4$, raising the concentration to 20 mM: the resting potential changed from 58 ± 12 to 58·5 ± 12 mV, (mean and S.D.; $n = 100$); this was not significant.

Ammonium and glutamate ions

Glutamates are involved in ammonia metabolism in cerebral tissues and modify their potassium content (see Discussion). 20 mM-sodium L-glutamate caused a significant fall in resting potentials, averaging 26% in the experiments of Table 3. After the initial incubation in 4 experiments, the glutamate-containing saline was replaced by the normal control saline, and an increase in potential ensued. By contrast, 20 mM-sodium D-glutamate was without significant effect on the resting potential.

The addition of ammonium chloride to make the concentration of ammonium salts in the media 5 mM was also without effect on resting potentials (Table 3). The ammonia content of the medium was determined at the end of the experiments in case it had volatilized owing to gas passing over it, but no loss was found.

In 6 experiments diminution of resting potential occurred with addition of 5 mM-NH_4Cl together with 20 mM-Na L-glutamate. This change was not significantly different from that of L-glutamate alone, but recovery was inhibited (Table 3).

TABLE 3. D- and L-glutamates and ammonium salts on resting potentials in guinea-pig cerebral cortex

Experimental arrangement as in Table 1B, except that the saline in the second period contained the additional substances indicated. Results from several experiments, each carried out with tissue from a different animal, are included in mean values (mV), followed by S.D. and number of penetrations.

	Potentials during successive periods				*P* value for change	
	First period normal saline	Second period		Third period normal saline	First to second period	Second to third period
No. of expts.		Addition				
5	58 ± 14 (130)	20 mM L-glutamate	44 ± 16 (130)	53 ± 15 (100)	0·001	0·001
3	53 ± 9 (75)	20 mM D-glutamate	53 ± 10 (75)	52 ± 10 (75)	Neither significant	
5	56·5 ± 13 (125)	5 mM-NH_4Cl	56 ± 11 (125)	56 ± 10 (125)	Neither significant	
4	54 ± 11·5 (150)	5 mM-NH_4Cl and 20 mM-L-glutamate	46 ± 9 (150)	45 ± 8 (100)	0·001	Not significant

DISCUSSION

Magnitude of potentials recorded from slices under normal conditions

The recording of intracellular resting membrane potentials is subject to extraneous effects from tip potentials (Adrian, 1956), from temporary blocking of electrodes and from damage to the cells on impalement (Woodbury, Hecht & Christopherson, 1951; Frank & Fuortes, 1955; Haapanen, Kolmodin & Skoglund, 1958). In the present investigation electrodes with large tip potentials, or with increased resistance, which is usually due to blockage by tissue, were discarded. On the assumption that rapidly depolarizing cells have been badly damaged by impalement, we rejected those potentials lasting less than 0·1 sec. Haapanen *et al.* (1958) concluded that cells with a surface area of 20,000μ^2 or less are unlikely to have the whole of their membrane potential recorded, owing to short-circuiting at the damaged membrane. The area quoted would

represent a spherical cell of 80μ diameter, and the tissue employed in the present study does not contain cells as large as this. A small, transient reading would also be expected on moving the electrode through a dendrite, an axon, or a small part of a cell.

The potentials recorded from the slices average 59 ± 12 (S.D.) mV, a value substantially higher than that of Li & McIlwain (1957). This is largely because the earlier study took account of every negative potential which could be recorded, whereas the present study is based on the maximum stable potential observed in each penetration. It is therefore understandable that the present values are more comparable with those of Phillips (1956), who recorded from Betz cells of cat cortex *in vivo* and found resting potentials between 48 and 69 mV. Phillips (1956) also recorded many values of 60–90 mV in 'inexcitable' cells, which he supposed were neuroglia. We have, as yet, not investigated whether the cells from which recordings were made respond to stimuli, or are neurones or neuroglia (see Tasaki & Chang, 1958).

It was usual, in those of the present experiments which were carried out under normal conditions and gave an average potential of 59 mV, to record some potentials of 80 mV or more. Possibly these higher values represent the less damaged cells, an assumption supported by Phillips's (1956) finding of a larger overshoot on stimulating cells of higher resting potentials. It did not seem possible to relate the larger potentials to a particular type of cell. Thus there did not appear to be a correlation between the potential of polarized elements and the distance travelled by the micro-electrode while remaining at a uniform negative potential. Presumably cells of a given type can be penetrated more or less centrally and from different angles, and thus present regions of negative potential to the electrode for a variety of different distances of travel (see p. 268).

It is to be emphasized that the polarized elements in the tissue *in vitro* are not sparse, but that the electrodes were in regions of negative potential for an average of 26% of their travel in the tissue, a value comparing favourably with observations *in vivo*. Much of the damage incurred in preparing the tissue presumably remains superficial or is made good during incubation. From a metabolic point of view the energy-rich phosphates of the tissue and its ionic composition, which suffer large changes during preparation for *in vitro* experiments, have been demonstrated above to be largely restored during the first 20–30 min of incubation.

Potassium salts

When the potassium concentration of the fluid bathing the tissue was increased above its normal value of 6·2 mM, tissue potentials diminished; diminution is to be expected from the Nernst equation, if the

cells were acting as potassium electrodes. Below 6·2 mM-K^+ tissue potentials also fell and could not be recorded in media of 1 mM-K^+, when the tissue contained 44 μequiv K/g (content in normal media was 60 μequiv/g). This is consistent with Pappius & Elliott's (1956) observation on cerebral slices in media of varying K^+ content, which showed that the maximum difference between internal and external K^+ concentration also occurred with media of 5–10 mM-K^+.

Potassium salts are required in incubating media if isolated cerebral tissues are to respond metabolically to applied electrical pulses (Cummins & McIlwain, 1961). Respiratory response to applied pulses increased nearly linearly with the potassium content of the tissue between 9 and 56 μequiv K/g fresh wt. of tissue. This implies an appreciable respiratory response in tissues in which we have been unable to record resting potentials in the present study. It remains likely that respiratory response to pulses depends on depolarization of cellular structures; the experimental arrangements in measuring the respiratory response aim at making the stimulus effective at all such structures whereas, as described above, it is likely that the micro-electrodes measure potentials in only the larger cellular structures.

Addition of 30–100 mM K salts to the fluid environment of cerebral tissues during metabolic experiments increases respiration (Ashford & Dixon, 1935; Dickens & Greville, 1935) and diminishes tissue phosphocreatine (Gore & McIlwain, 1952). The depolarization which appears likely to be intermediary in these actions of potassium salts is clearly demonstrated in the present experiments. Release and diffusion of potassium salts from cerebral tissues *in vivo* have been considered an important part of the mechanism involved in the spreading depression of Leão (1944) by Grafstein (1956) and by Bureš (1956). Taking the intracellular concentration of potassium salts *in vivo* as 100 mM and the extracellular space as 40% (Pappius & Elliott, 1956), accumulation extracellularly of some 12% of the tissue potassium would be needed to produce the 30 mM-K^+ now associated with depolarization. Loss of this magnitude was found by Cummins & McIlwain (1961) to be caused by 2 min electrical stimulation of the isolated tissue. Graftstein (1956) quotes calculations of A. L. Hodgkin suggesting that the speed of spread of the inexcitable condition is consistent with the speed of diffusion of potassium salts.

The fall in resting potential observed to follow the addition of L-glutamic acid to cerebral tissues is parallelled in spinal motoneurones of lightly anaesthetized cats (Curtis, Phillis & Watkins, 1960). Of known properties of L-glutamic acid, that most directly related to its action in diminishing resting potentials appears to be its effect on potassium salts. Although cerebral tissues incubated in media with glucose and glutamate reach

higher levels of potassium salts, per gram fresh weight of tissue, than those in media with glucose only (Terner, Eggleston & Krebs, 1950), Pappius & Elliott (1956) observed the tissue with glutamate to swell to such an extent that the computed intracellular potassium concentration fell by some 15%. Cerebral tissues swell in several situations in which energy-yielding reactions fail, and glutamate leads to fall in the phosphocreatine of cerebral tissues (McIlwain, 1952), giving the impression that energy-consuming reactions are increased with glutamate. These processes could include the assimilation of glutamic acid itself, which reaches tissue concentrations much above those in the medium (Stern, Eggleston, Hems & Krebs, 1949) and the synthesis of glutamine (Weil-Malherbe, 1936; R. J. Woodman & H. McIlwain, unpublished); presumably such processes consume sufficient energy-rich intermediates to deplete the supply to ion-transport mechanisms.

Sodium and other ions

The present investigations appear to give the first demonstration of requirement by mammalian cerebral tissues, for the presence of sodium salts for maintenance of resting membrane potentials. The presence of sodium salts has already been shown to be necessary in obtaining metabolic response to electrical stimulation of mammalian cerebral cortex (Gore & McIlwain, 1952).

In seeking a reason for this it appears important that deficiency in the Na of incubating media in the present experiments, as in those of Pappius, Rosenfeld, Johnson & Elliott (1958), led to fall in the K of cerebral tissues. Lowered external Na has been shown also to decrease the K content of desheathed sciatic nerves from the frog (Shanes, 1956). Whether this implies requirement for Na directly in the active transport of K is not answered by the present experiments; a less direct relationship is favoured by the fall in the phosphocreatine of the tissue found to occur in Na-free media (Gore & McIlwain, 1952). In the present experiments the effects of incubating in Na-free media were not readily reversible; if, when potentials did not reappear in the Na-free media, NaCl was added, the tissue K did not increase nor did potentials reappear. This was not due to osmotic effects or to direct toxic action on the part of the choline, which was then the main cation; potentials were still observable in tissues incubated in media with 300 mM-NaCl, and in media containing 125 mM choline chloride and 25 mM-$NaHCO_3$.

Absence of Ca or of Mg salts from media in which cerebral tissues were incubated adversely affected both metabolic characteristics and the establishment of resting potentials in the tissue. In the depleted media tissues resynthesized only some 60% of their normal phosphocreatine (Gore & McIlwain, 1952). In the present experiments such media led to

a delay of 30–40 min in the establishment of resting potentials, though when potentials appeared they were close to normal values. In these experiments the tissue itself contributes some Ca and Mg to the system.

The experiments as a whole show considerable scope for investigation of electrophysiological phenomena in isolated mammalian cerebral tissues. The system offers the usual advantages of an *in vitro* technique in allowing experimental changes much wider than are permissible *in vivo*; the complete replacement of Na salts gives an example of this.

SUMMARY

1. Resting membrane potentials can be recorded intracellularly in slices of mammalian cerebral cortex maintained at the surface of oxygenated glucose salines. The average potential recorded was 59 ± 12 mV (S.D.) and thus approximates to values previously observed *in vivo*. Some 20–30 min incubation was needed for the reappearance of the potentials *in vitro*.

2. The potentials were dependent on the potassium concentration of the saline, maximum potentials being recorded at an external potassium concentration of 6 mM. After establishing potentials in such media, additions of KCl to 15–50 mM caused depolarization of the tissue; potentials could subsequently be re-established in media with 6 mM-K^+.

3. No change in resting potential was detected when the external sodium concentration was altered between 25 and 300 mM, but media without sodium salts did not permit repolarization of the tissue.

4. Magnesium and calcium concentrations could be varied considerably without altering the membrane potentials, but their absence caused a 30–40 min delay in repolarization on incubating the cerebral slice.

5. Addition of 5 mM ammonium chloride did not affect cell polarization. 20 mM L-glutamate decreased the resting potentials, while 20 mM-D-glutamate had no effect.

6. The observations are discussed in relation to metabolic properties of cerebral tissues and to resting membrane potentials observed in other systems.

We wish to thank those mentioned in the text who contributed to this study; at different times during the investigation, technical help has been given by Mr J. G. Platt, Miss M. Juster, and Miss S. Johnson, which we acknowledge with gratitude. We are also grateful to Dr J. E. Houldin, Dr A. E. Maxwell and Dr J. F. Palmer for helpful discussion and advice, to Dr R. D. Keynes for his comments on the manuscript, and to the Medical Research Council for support.

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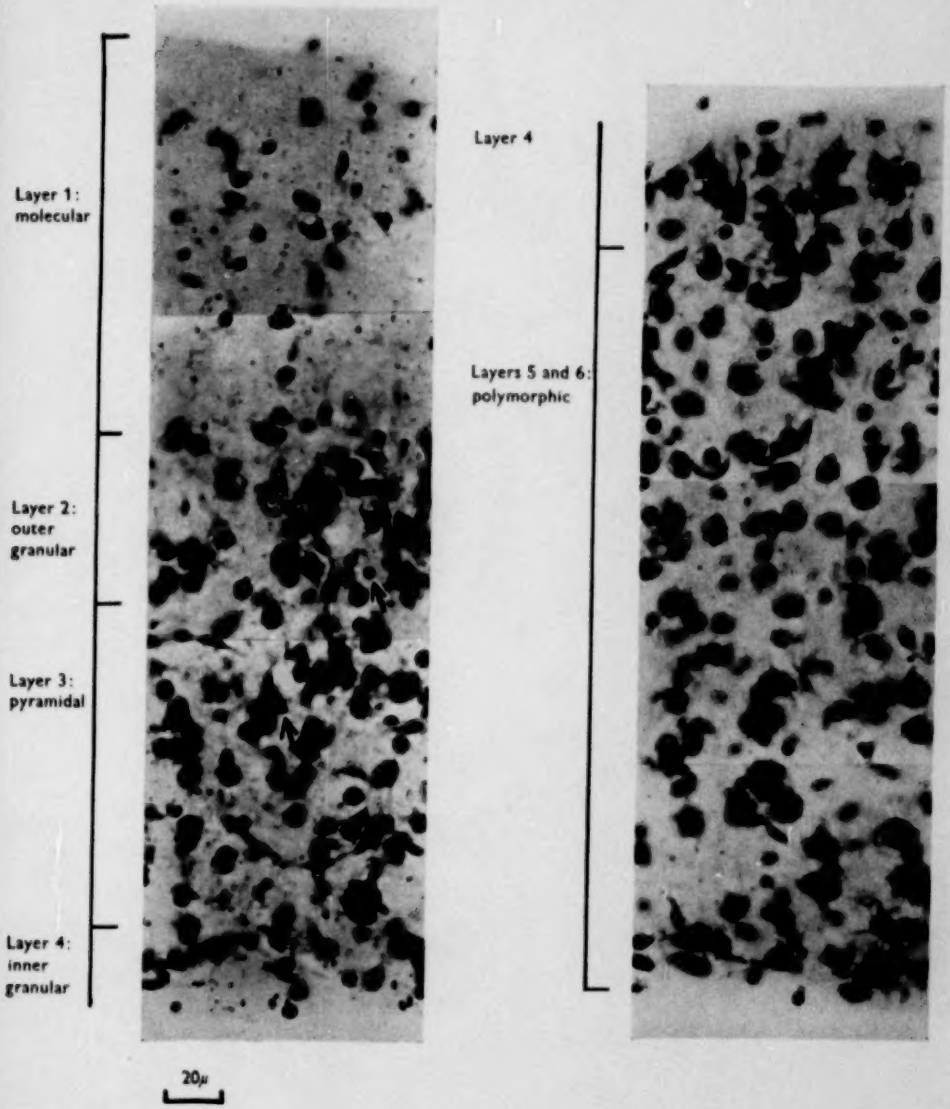
EXPLANATION OF PLATE

Photomicrographs of stained sections prepared from slices of parietal cortex of guinea-pig used in the present experiments. Slices were fixed in 10% formalin, embedded in celloidin (Carleton, 1938) cut at 30μ, and stained with cresyl violet (Spielmeyer, 1930). Left; outer slice cut first from the convexity of the hemisphere; right, slice cut second. The upper arrow in the first slice shows a neuroglial cell, the lower a neurone. Horizontal scale, 20μ; the lamina noted. Preparation by Dr J. B. Brierley.



H. H. HILLMAN AND H. McILWAIN

(*Facing p.* 278)

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TRANSFER OF GLUCOSE AND FLUID BY DIFFERENT PARTS OF THE SMALL INTESTINE OF THE RAT

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The concept of gradients of activity by the intestine was put forward by Alvarez (for reference see Alvarez, 1939), who thought that a number of properties of the intestine could be explained by gradually diminishing activities of various kinds along the intestine, beginning from the jejunal end. Alvarez was interested primarily in the motor activities of the intestine, but later studies on the rat intestine *in vitro* by Fisher & Parsons (1949*b*), Wilson & Wiseman (1954) and Smyth & Taylor (1957) have supported the idea of different intestinal regions showing different levels of activity, at least as regards O_2 consumption, glucose transport and fluid transfer, and in general these activities were found to be greater in the jejunal than the ileal regions. The following experiments were undertaken to investigate in more detail the transfer capacity of different parts of the intestine. Glucose and fluid transfer were selected for study, as these are known to vary in different intestinal regions, and one object was to study the correlation between glucose and water transfer in different parts of the intestine. A preliminary account has been published by Barry, Matthews & Smyth (1960).

METHODS

The intestinal preparation used was the everted sac of small intestine of the rat as described by Wilson & Wiseman (1954). White rats were anaesthetized with pentobarbitone sodium, the whole of the jejunum and ileum removed, transferred to 0·9% NaCl at room temperature and everted. (The duodenojejunal junction is arbitrarily taken as the point where the gut is closely bound to the posterior abdominal wall, and the jejunum was divided as close to this as possible). After eversion the intestine was returned to 0·9% saline. In order to facilitate the identification of different parts it was found useful at this stage to place ligatures of different colours on the jejunal and ileal ends. The everted intestine was then transferred to a Perspex trough, 1 m long, containing 0·9% NaCl. The bath was constructed by dividing longitudinally a Perspex tube of 1 in. (2·5 cm) diameter. The ligatures at the ends of the intestine were fixed to hooks and the intestine stretched until it was just straight. It was then further stretched until the total length in centimetres was the next multiple of five. Usually the extended length was 65–75 cm. With the metre scale alongside, the intestine was now divided by ligatures into five equal parts, and sacs were made from these five parts. The sacs were filled with 1 ml. bicarbonate saline (Krebs & Henseleit, 1932) with or without glucose, suspended in 15 ml. of the same solution, and

shaken in flasks as described by Parsons, Smyth & Taylor (1958). As five sacs of intestine were used, and as these could not be made simultaneously, a system had to be devised to eliminate the factor of the order in which the sacs were made, as Fisher & Parsons (1949*a*) had stressed the importance of minimal delay in setting up *in vitro* intestinal preparations. The following scheme was used. Preliminary experiments showed that if the segments were taken out of the trough at 6 min intervals this allowed adequate time for dealing with each in turn, and hence this procedure was adopted as a routine. When this is done, however, the last segment to be used must remain 24 min longer in the saline than the first before it is used. If the experiments are done in sets of five the different segments of intestine can be used in different orders, so that in a complete set all will have received the same treatment. An example is shown in Table 1. The five segments of intestine are numbered I, II, III, IV and V starting from the jejunal end. In the first experiment of the set the sacs were made in the order I, II, III, IV and V; in the second they were made in order II, III, IV, V and I; in the third III, IV, V, I and II and so on for a set of five experiments. When the set of five experiments is completed the average values can be obtained either (*a*) for the sac made first, the sac made second, etc., or (*b*) for sac I, sac II, sac III, sac IV and sac V. In the first case the average values will include in each case figures for sac I, sac II, sac III, sac IV and sac V, and therefore will all be comparable. In the second case the average values will include figures for the sac made first, second, third, fourth and fifth and will again be comparable. If a larger number of results are required average figures can be taken from two or more sets each consisting of five experiments. In this way it is possible to study the effect both of order of making the sac and also the part of the intestine used. It should be noted that, while the sacs were waiting to be used, they were kept in 0·9% NaCl solution at room temperature with no added glucose and were not oxygenated. Preliminary experiments rather surprisingly did not show that more consistent results were obtained with more elaborate conditions of maintenance, but this point is being further investigated. The methods of calculating the results were as described by Parsons *et al.* (1958), and the terms used in describing these were as used by Jervis & Smyth (1960). For convenience of the reader they are repeated here. The fluid in which the sacs were suspended is called the mucosal fluid, and the fluid inside the sac the serosal fluid. The mucosal fluid transfer is the diminution in the volume of fluid on the mucosal side during the course of the experiments; the serosal fluid transfer is the increase in volume inside the sac; the gut fluid uptake is the increase in the volume of fluid in the gut wall; the initial mucosal and serosal glucose concentrations are the concentrations in the mucosal and serosal fluid at the beginning of the experiment; the final mucosal glucose concentration is the concentration in the mucosal fluid at the end of the experiment; the final serosal concentration is the concentration of glucose in the serosal fluid at the end of the experiment; the final concentration gradient is the difference between the final serosal glucose concentration and the final mucosal glucose concentration; the mucosal glucose transfer is the amount of glucose which disappears from the mucosal fluid; the serosal glucose transfer is the increase in glucose in the serosal fluid; the gut glucose uptake is the difference between the mucosal glucose transfer and the serosal glucose transfer (the term includes therefore glucose metabolized and also glucose present in the gut wall at the end of the experiment); the mucosal concentration transferred is the mucosal glucose transfer divided by the mucosal fluid transfer; the serosal concentration transferred is the serosal glucose transfer divided by the serosal fluid transfer. All these quantities as defined are taken to be positive. Under certain conditions some of them may become negative. One other measurement which has been used by Wiseman (1955) is the final concentration ratio developed, by which is meant the ratio of the final concentration in the serosal fluid of substance transferred to its final concentration in the mucosal fluid.

Glucose was estimated by the method of Nelson (1944), as modified by Somogyi (1945).

RESULTS

Effect of order of making the sacs

This was studied in a set of five experiments in which sacs were made in different orders as described. Bicarbonate saline was used, with glucose added in an initial concentration of 500 mg/100 ml. The initial mucosal and serosal solutions were the same. The results of one set of experiments are seen in Tables 1 and 2, which give the mucosal fluid transfer and mucosal glucose transfer. The tables show the range of variation obtained

TABLE 1. Mucosal fluid transfer by sacs of everted intestine in five experiments in which the sacs were made in different order. The sacs are numbered from the jejunal end. The average of the figures in the first vertical row will be the value for sac I; the average of the bold figures in the oblique row will be the value for the sac made first

	Mucosal fluid transfer (ml./g initial wet wt./hr)				
Order of making sacs	Sac I	Sac II	Sac III	Sac IV	Sac V
I, II, III, IV, V	**1·42**	0·92	1·25	1·22	0·42
II, III, IV, V, I	1·26	**1·46**	1·34	0·76	0·75
III, IV, V, I, II	0·96	1·00	**1·94**	1·51	1·30
IV, V, I, II, III	1·10	1·25	1·64	**1·55**	1·19
V, I, II, III, IV	0·97	1·40	1·19	0·59	**0·93**

TABLE 2. Mucosal glucose transfer by sacs of everted intestine in five experiments in which the sacs were made in different order. The sacs are numbered from the jejunal end

	Mucosal glucose transfer (mg/g initial wet wt./hr)				
Order of making sacs	Sac I	Sac II	Sac III	Sac IV	Sac V
I, II, III, IV, V	28·22	25·92	31·07	21·50	3·78
II, III, IV, V, I	21·39	31·14	33·03	15·90	1·92
III, IV, V, I, II	18·68	23·96	39·79	18·21	3·76
IV, V, I, II, III	26·49	32·15	38·95	30·54	5·03
V, I, II, III, IV	26·77	31·69	26·79	11·99	4·31

in a complete set of experiments carried out in this way, and they also, even at a casual inspection, suggest that there are differences in transfer capacity of different parts of the intestine.

The differences due to the order in which the sacs were made were determined by carrying out an additional set of experiments, and from the two sets of experiments average values were taken for the ten sacs made first, the ten made second, third, fourth and fifth. The results are seen in Table 3. The results for both glucose and water transfer are expressed both as the transfer per gram initial wet weight of tissue and also as a percentage of the sacs made first. There is evidence of some deterioration in transfer capacity for both glucose and water due to delay in making the sacs. The significances of the difference between the means for the sacs made first and fifth are for glucose transfer $P < 0{\cdot}1$, and for fluid transfer

$P < 0.02$. There is thus a significant difference for fluid transfer but not for glucose transfer. Although in the case of glucose the difference is not significant, the results do not prove that there is no difference, and it would obviously be unwise to use the sac made first and last to compare directly transfer capacity. Hence in comparing the activities of different parts of the intestine it is safer to use the average values from sets of experiments in which the sacs are made in different orders.

TABLE 3. Effect of order of making sacs on the mucosal transfer of fluid and glucose. The table shows (*a*) the average values with standard errors (for two sets of five experiments each) of the mucosal transfer in the sac made first in each experiment, the sac made second in each experiment, etc., and (*b*) the average values expressed as a percentage of the average values of the sac made first

	Order of making sacs				
	First	Second	Third	Fourth	Fifth
Average mucosal glucose transfer expressed as:					
(*a*) mg/g wet wt./hr	26·99 ± 4·13	23·06 ± 3·65	20·73 ± 4·01	20·49 ± 3·15	19·47 ± 3·29
(*b*) percentage of sac made first	100	85·4	76·80	75·91	72·13
Mucosal fluid transfer expressed as:					
(*a*) ml./g wet wt./hr	1·51 ± 0·12	1·28 ± 0·11	1·09 ± 0·09	0·98 ± 0·08	0·97 ± 0·14
(*b*) percentage of sac made first	100	84·8	72·2	64·9	64·2

Transfer of glucose and fluid in different parts of the intestine

This was studied by taking average values for twelve parameters from the same two sets of experiments (i.e. 10 values for each part of the intestine), in which the sacs were made in varying orders; and the results are given in Tables 4 and 5.

As regards fluid transfer (Table 4) the middle segment of the intestine is seen to be the most active as measured by mucosal transfer, serosal transfer or gut uptake. If we take mucosal transfer, the significance of the superiority of sac III over any other sac is indicated by the following values of P, < 0.01 for sac I, < 0.1 for sac II, < 0.02 for sac IV and < 0.001 for sac V. The difference between the other sacs is scarcely or not significant. It will be noted in particular that sacs I and V are very similar, although it will be shown later that there is an important difference between them.

The details for glucose transfer are seen in Table 5. The first four segments all show removal of glucose from the mucosal fluid (mucosal glucose transfer) and a transfer of part of this to the serosal fluid (serosal glucose transfer). A large part of the mucosal transfer is retained in the gut wall (gut glucose uptake), the fraction for the first four segments being respectively 0·85, 0·81, 0·71 and 0·80. In the case of the fifth segment not only is the total mucosal transfer retained, but some glucose disappears from

the serosal fluid also. Most of the glucose retained in the gut wall is presumably metabolized, as was shown by Parsons *et al.* (1958). As a result of the movement of glucose the final serosal concentration is greater than the final mucosal concentration in the first four sacs, i.e. in the terminology used there is a positive final concentration gradient. If the ratios of the final concentrations are used as a comparison the final concentration ratio

TABLE 4. Fluid transfer and uptake by everted sacs made from different parts of the intestine. The figures are the average values with standard errors for 10 sacs (2 sets of five experiments) made from each part of the intestine. The sacs are numbered from the jejunal end

	Sac I	Sac II	Sac III	Sac IV	Sac V
	(ml. fluid/g initial wet wt. intestine)				
Mucosal transfer	1·03 ± 0·10	1·21 ± 0·12	1·55 ± 0·11	1·07 ± 0·12	0·96 ± 0·10
Serosal transfer	0·80 ± 0·10	0·82 ± 0·09	1·09 ± 0·11	0·71 ± 0·11	0·60 ± 0·09
Gut uptake	0·23 ± 0·03	0·39 ± 0·04	0·46 ± 0·02	0·36 ± 0·06	0·36 ± 0·06

TABLE 5. Glucose transfer capacity of different parts of the intestine. The figures are the average values with standard errors (two sets of five experiments each) from different parts of the intestine. Transfers are expressed per gram initial wet wt. of intestine. The sacs are numbered from the jejunal end

	Sac I	Sac II	Sac III	Sac IV	Sac V
Initial mucosal and serosal concn. (mg/100 ml.)	500	500	500	500	500
Final mucosal concn. (mg/100 ml.)	350 ± 7·8	281 ± 17	283 ± 17	408 ± 7	504 ± 8
Final serosal concn. (mg/100 ml.)	492 ± 25	595 ± 18	723 ± 27	495 ± 41	155 ± 16
Final concn. gradient (mg/100 ml.)	141 ± 26	314 ± 26	441 ± 37	87 ± 47	−349 ± 20
Final concn. ratio	1·41 ± 0·07	2·19 ± 0·15	2·67 ± 0·20	1·23 ± 0·12	0·31 ± 0·03
Mucosal glucose transfer (mg/g)	23·65 ± 1·4	29·9 ± 1·8	3·43 ± 1·6	18·7 ± 1·7	4·3 ± 1·0
Serosal glucose transfer (mg/g)	4·0 ± 0·7	5·8 ± 0·7	10·1 ± 1·2	3·8 ± 1·0	−2·5 ± 0·40
Gut glucose uptake (mg/g)	19·5 ± 1·2	24·1 ± 1·2	24·2 ± 0·8	14·9 ± 1·0	6·8 ± 1·0
Mucosal concn. transferred (mg/100 ml.)	2492 ± 272	2536 ± 135	2243 ± 63	1872 ± 176	480 ± 112
Serosal concn. transferred (mg/100 ml.)	476 ± 77	713 ± 43	922 ± 43	478 ± 138	−557 ± 117

is greater than unity in the first four sacs, and less than unity in the fifth sac. The changes in concentration in mucosal and serosal solutions will depend on the relative movements of fluid and glucose. In the first four sacs the concentration of glucose in the fluid leaving the mucosal side (mucosal concentration transferred) is much higher than the initial glucose concentration, and hence the final mucosal glucose concentration is lower than the initial concentration; in the fifth sac the concentration of glucose in the fluid leaving the mucosal side is lower than the initial concentration, and hence although some glucose leaves the mucosal side in the fifth sac the final concentration of glucose in the mucosal fluid is on the average slightly higher than the initial concentration, although not significantly so.

As regards the glucose concentration in the fluid transferred to the serosal side (serosal concentration transferred) only in sacs II and III was this higher than the initial glucose concentration, and hence in these sacs the final serosal glucose concentration is higher than the initial serosal glucose concentration. In the fifth sac fluid enters the sac contents while glucose leaves, and hence the serosal glucose concentration transferred appears as a negative quantity. Whatever criterion is used for transfer capacity of glucose, i.e. mucosal transfer, serosal transfer, final concentration gradient, final concentration ratio, final mucosal or serosal concentration, or gut uptake, sac III appears to be the most active, and sac V the least active. Sacs II and IV occupy an intermediate position between sac III and sacs I and V, respectively. The statistical significance of the differences will be apparent from the standard errors of the means included in the table.

TABLE 6. Mucosal transfer of fluid and glucose in different parts of intestine, expressed as a percentage of the values for sac III. The sacs are numbered from the jejunal end

	Sac I	Sac II	Sac III	Sac IV	Sac V
Mucosal fluid transfer	66	78	100	69	62
Mucosal glucose transfer	69	87	100	54	13

A study of Tables 4 and 5 indicates that although sac III has the greatest transfer capacity for both glucose and fluid and sac V the smallest, glucose and fluid transfers by no means run parallel. This is shown by comparing the relative transfer, taking the values for sac III as 100%, and these figures are given in Table 6, which shows that while mucosal glucose and fluid transfer run roughly parallel in the first three segments, the glucose transfer falls much more sharply in the last two than does fluid transfer.

Fluid transfer in the absence of glucose

These experiments were carried out in two sets of five exactly as described previously, except that no glucose was present. The results are shown in Table 7, which gives the average values for each fifth of the intestine from two sets of five experiments each. This shows that absence of glucose has a much greater effect on the jejunal end of the intestine than the ileal end. This is well seen by taking figures obtained in the absence of glucose and expressing them as a percentage of the figures obtained in the presence of glucose. The values obtained (starting from sac I) are 22, 26, 34, 56 and 70%. The only possible conclusion seems to be that there are two different processes associated with water transfer, one dependent on the presence of glucose; the other independent of it. By subtracting the values in the absence of glucose from those in the presence of glucose we can get values for the magnitude of water transfer dependent on glucose.

Figure 1 shows in diagrammatic form, the value of each of these types of mucosal transfer, and also the total mucosal transfer by different parts of the intestine. This diagram suggests that glucose dependent transfer is greatest at the jejunal end and declines towards the ileum, while glucose-independent transfer is very small in the jejunum and increases towards the ileum. The superior position of sac III in fluid transfer is probably due to the overlap of the two mechanisms in this part of the intestine.

TABLE 7. Transfer of fluid by sacs of everted intestine in the absence of glucose. The figures are the average values with standard errors (two sets of five experiments each) from different parts of the intestine. Transfers are expressed per gram initial wet wt. of intestine. The sacs are numbered from the jejunal end

	Sac I	Sac II	Sac III	Sac IV	Sac V
	(ml./g initial wet wt. intestine)				
Mucosal fluid transfer	0·23 ± 0·011	0·31 ± 0·009	0·53 ± 0·028	0·60 ± 0·020	0·67 ± 0·040
Serosal fluid transfer	0·13 ± 0·007	0·20 ± 0·006	0·31 ± 0·025	0·35 ± 0·011	0·32 ± 0·015
Gut fluid uptake	0·10 ± 0·007	0·12 ± 0·008	0·22 ± 0·005	0·25 ± 0·006	0·35 ± 0·012

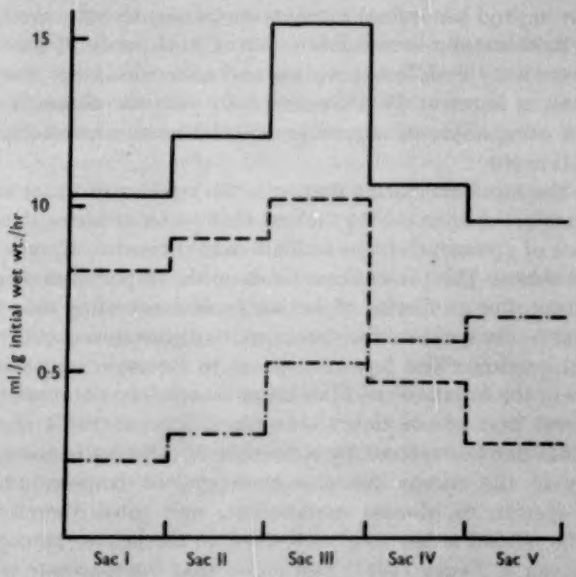

Fig. 1. Mucosal fluid transfer by different segments of the intestine. The three values are total fluid transfer (——), glucose dependent transfer (- - -) and glucose independent transfer (- · - · -). The glucose dependent transfer is calculated from the differences between the values for transfer in the presence and absence of glucose, and full details of these with the standard errors are given in Tables 4 and 7.

DISCUSSION

The results provide the most detailed information yet published of the activity of different parts of the rat intestine for glucose and water transfer. They include twelve different parameters of absorption expressed for five different intestinal regions. Although all these parameters are not independent, there is a strong case for taking all of them into account in investigating any problem of intestinal transfer *in vitro*, and we would suggest that data such as that included in Tables 4 and 5 should form an indispensable base line for studying any effects on intestinal transport *in vitro*. Failure to take any of these parameters into consideration might well result in fallacious deductions about the transfer capacity of the intestine. Possible sources of error are (1) the fact that mucosal transfer depends to a large extent on glucose metabolism as well as on glucose transfer, and hence metabolism will have a considerable effect on the final concentration gradient and final concentration ratio; (2) the fact that glucose and water transfer are related to different degrees in different parts of the intestine, and hence final concentrations may be influenced by either glucose or fluid transfer or a combination of both; and (3) the difference in transfer capacity in different regions and particularly the sharp change in passing from segment IV to segment V, with the consequent danger involved in using adjacent segments of intestine as controls in any particular experiment.

Perhaps the most interesting finding is the relation of water transfer to glucose transfer. Fisher (1955) showed that water transfer depended on the presence of glucose, and this result was confirmed by Smyth & Taylor (1957) and others. The present results show the importance of glucose in water transfer, but go further than this in demonstrating that the water transfer can be divided into two fractions, one glucose-dependent and one glucose-independent. The former appears to be more important in the upper part of the intestine, and the latter is certainly the more important in the lowest part of the ileum. Smyth & Taylor (1957) showed that glucose could not be replaced by a number of other substances, and this suggests that the energy for glucose-dependent transfer comes from reactions specific to glucose metabolism, and must therefore almost certainly be related either to glycolysis or to the hexose monophosphate shunt. Smyth & Taylor (1957) also found that fluoroacetate was not an effective inhibitor of fluid transfer, and, as Gilman & Koelle (1960) have pointed out, this is in favour of the energy utilized not coming from the citric acid cycle.

In contrast to the glucose-dependent transfer it would seem probable that the glucose-independent transfer depends on energy from the citric

acid cycle. Preliminary experiments have in fact suggested that fluoroacetate abolishes the glucose-independent transfer and this would be consistent with this hypothesis.

The results are also of interest in relation to the concept of gradients of activity in the intestine. It is clear that there are marked regional differences in transfer capacity in the intestine, but there is not a regular variation in passing along the intestine. Glucose transfer is greatest in the middle fifth, and smallest in the last fifth. Total fluid transfer is also greatest in the middle fifth, but when the two types of fluid transfer are separated, the glucose-dependent is not very different in the upper three segments, but much smaller in the lowest segment, while the glucose-independent fluid transfer is certainly greatest in the lowest fifth and much smaller in the upper part of the intestine. The local regional differences in both glucose and fluid transfer could obviously be missed by studying long segments of intestine and indeed for this reason Fisher & Parsons (1949*b*) thought that glucose transfer decreased along the intestine on approaching the ileo-caecal valve, and Smyth & Taylor (1957) drew a similar conclusion about water transfer. In both cases the long length of intestine used masked the local differences revealed in the present experiments. It is of course also possible that if the intestine were divided up into still smaller lengths other local differences would be seen. Just as this paper is being prepared for press the results of Clarkson & Rothstein (1960) have appeared, in which transport in four segments of intestine has been studied from a rather different point of view. Transport cannot be directly compared in the two sets of experiments, but our results are certainly compatible with those of Clarkson & Rothstein.

Another result of some interest is the effect of delay in making the sacs, and this seems to be much less than might have been supposed. Fisher & Parsons (1949*a*) stressed the importance of avoiding any delay in interrupting the oxygen supply of the tissues, and believed the success of the *in vitro* preparation depended on this. It is clear that with everted sacs the urgency of continuous oxygen supply is much less, and sacs remaining in unoxygenated saline for 24 min at room temperature do not lose their capacity to transport either glucose or water.

SUMMARY

1. A technique is described for studying glucose and water transfer capacities of different parts of the intestine, so as to eliminate the factor of delay in making sacs from these different parts.

2. The capacity of the rat intestine for transfer of glucose and water *in vitro* is expressed in 12 parameters for five different parts of the

intestine. The capacity for glucose and water transfer varies in different regions of the intestine and the middle section is most active for both glucose and water transfer. Glucose and water transfer do not run parallel and the ileal end has very little capacity for glucose transfer, but considerable capacity for water transfer.

3. The water transfer can be divided into two fractions, one glucose-dependent and one glucose-independent. The former is more important in the jejunum, the latter in the lower ileum, but there is a considerable overlap of the two water-transfer mechanisms in the middle part of the intestine.

4. It is suggested that the energy for glucose-dependent transfer comes from either glycolysis or the hexose monophosphate shunt, while the energy for glucose-independent transfer comes from the citric acid cycle.

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RUBIDIUM AND CAESIUM ENTRY, AND CATION INTERACTION IN FROG SKELETAL MUSCLE

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When a muscle is exposed to a solution containing Rb or Cs ions there is a gradual replacement of its K by the foreign ion (Lubin & Schneider, 1957; Sjodin, 1959). The aim of this paper is to present the kinetic results for the interchange of Rb or Cs and K, using a model proposed to explain the observed dependence of K-for-K exchange on external concentration (Harris & Sjodin, 1961). By 'interchange' is meant the equivalent-for-equivalent replacement of an ion by a chemically unlike ion; this process can be followed by analytical methods. By 'exchange' is meant the equivalent-for-equivalent replacement of an ion by a chemically identical ion; this can only be followed by the use of isotopically labelled material. Net movement of a cation can take place either as a consequence of interchange with a different cation, or along with a mobile anion. The model used allows uptakes from K-free solutions and from mixtures to be treated consistently. Values for the relative exchangeabilities of K with itself and with Rb and Cs are obtained.

METHODS

The uptakes of ^{42}K or ^{86}Rb or ordinary Rb were measured, using the sartorii of *Rana temporaria*. The Cs results are taken in part from Sjodin (1959), who used *Rana pipiens*. The tracer experiments were made by the technique described by Harris & Sjodin (1961); a series of readings of the radioactivity of the tissue was obtained after timed exposures to the radioactive solution. Before the measurements the muscles were blotted on filter paper moistened with an inactive solution chemically similar to the soak solution. At the end of each experiment the muscle was extracted overnight in dilute nitric acid and the extract used for analysis by flame photometry. In making most of the experiments depending on Rb or Cs analysis after timed exposure to a given solution, it was found convenient to apply a 1 min wash in Na methyl sulphate solution (0·11 M) at 0° C before extraction of the cations; this wash would remove much of the extracellular solution and so render less necessary a correction for the trapped Rb or Cs.

The analyses were made with a flame spectrophotometer using a prism monochromator, Beckman oxy-hydrogen burner and a photomultiplier tube with sensitivity extending beyond 8500 Å (E.M.I. type 9553B). It was confirmed that at the levels used the Rb–K

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interference was negligible. There was, however, some K in the Rb salts; this amounted to 1 part K to 50 Rb in the RbCl and 1 part K to 150 Rb in the Rb_2CO_3. The CsCl contained 1 part K to 250 Ca.

The experiments were started in solutions made up with mixtures of the alkali chlorides. These mixtures had [NaCl] = 80 − ½[RbCl]; [$NaHCO_3$] = 30 mM; [KCl] and [RbCl] as stated and [$CaCl_2$] = 2 mM. Up to Rb concentrations of 40 m-equiv/l. provided the K concentration did not exceed 5 m-equiv/l. there was little or no net gain of cation from the solutions; the sum (K + Rb) found after 4 hr immersion was between 75 and 97 m-equiv/kg, while the K content of fresh muscles was 75–85 m-equiv/kg. Hence in these cases it is justifiable to regard the Rb^+ (and labelled K^+) entry as occurring mainly in exchange for the muscle K^+. This was not so when 80 m-equiv Rb/l. was used, for then the (Rb + K) increased to about 130 m-equiv/kg tissue, presumably on account of entry of RbCl (and KCl when K was present). For this reason the later Rb experiments were all made in Cl-free mixtures of $RbHCO_3$ and K and Na methyl sulphates ($NaMeSO_4$). These had

$$[K + Rb + Na] = 115 \text{ m-equiv/l.}, \quad [HCO_3] = 20 \text{ m-equiv/l.}$$

or equal to [Rb] when [Rb] exceeded 20; [methyl sulphate] = 115 − [HCO_3] m-equiv/l.; [Ca acetate] = 2 mM. The use of high concentrations of $RbHCO_3$ equilibrated with 95 % O_2 + 5 % CO_2 led to high pH (8·3 when $RbHCO_3$ = 80 mM) but the K exchange results already described (Harris & Sjodin, 1961) show that in alternative solutions containing either high or normal [HCO_3] the K exchange follows the same course. This is the case despite the precipitation of calcium in the high bicarbonate solution. Confirmation that pH and Ca have little effect on the Rb entry was obtained by comparing the results obtained in the two kinds of solution, namely with and without Cl. The caesium salt solutions were made from mixtures of CsCl or $CsNO_3$ with Na salts and had [Cs + Na] = 120 m-equiv/l.

Some measurements of the space accessible to the dye naphthol green were made at the same time as certain analytical experiments by adding 0·5 % of the dye to the solution. The muscle extracts were then made for 30 min in distilled water without added acid and the dye contents were read on a colorimeter. The extracts were then returned to the vessels containing the muscles and a drop of nitric acid added to assist cation release. The space measured in relatively fresh muscles (up to 30 min in isolation) was 0·11 ± 0·02 ml./g (S.D. for 11 results); after longer isolation values tended to be higher, for example, 0·14 ml./g after 2 hr.

RESULTS

Method of presentation

Penetration of the cations into the cell is assumed to occur through an outer compartment which has ion-exchange properties (Harris & Sjodin, 1961). In the absence of net movement accompanying chloride ions, the cations only move by exchange diffusion (Ussing, 1949) against ions already adsorbed. The equilibration of the compartment bounded by the ion exchange region then occurs as if from a source having a time-dependent concentration of the new ions deriving from the solution. Suppose a constant concentration $[Rb]_e$ is applied to the ion exchanger and no appreciable external concentration of the desorbed K ions is allowed to build up (by use of a large volume of solution) the equation governing the rate of exchange of adsorbed ions is

$$dRb_a/dt = k_{Rb}[Rb]_e(N - Rb_a). \qquad (1)$$

Here Rb_a denotes the activity of the adsorbed Rb per unit mass of tissue and N is the total activity of sites able to hold Rb ions, so $(N\text{-}Rb_a)$ is the activity of the still unexchanged K ions in the adsorption region. k_{Rb} is the rate coefficient for Rb-K interchange.

The integral form of this equation for the condition that $Rb_a = 0$ at $t = 0$ is

$$Rb_a/N = \{1 - \exp(-k_{Rb}[Rb]_e t)\} \quad (2)$$

in which N can be replaced by Rb_a ($t = \infty$) or by K_a ($t = 0$), provided that eventually all the adsorbed K is replaceable by Rb. This is an exponential process of build-up within the outer region with a rate constant equal to $k_{Rb}[Rb]_e$, that is, proportional to $[Rb]_e$. If, next, the outer region be regarded as the source from which diffusion into the cell interior occurs and provided the disturbance of the exponential process due to passage of ions from the cell interior is negligible, one can compare the course of the uptake into the cell interior with that of the appropriate diffusion process. When the rate constant (β) of equilibration of the source (the outer region) is so low that the quotient $a^2\beta/D < 1$, a being the radius of a cylindrical object or the half thickness of a sheet and D the internal diffusivity, then the shape of the object and the values of a and D become unimportant (Harris & Sjodin, 1961) and the fractional equilibration of the interior depends almost entirely on the value of βt. Since on our assumptions the rate constant β is identified with $k_{Rb}[Rb]_e$ we should find Rb uptakes from all K-free Rb solutions falling on a single curve when plotted against $[Rb]_e t$ or a function of this product. The omission of the factor k_{Rb} from the function used as abscissa corresponds to multiplication of the derivative of the uptake with respect to the abscissa by the appropriate function of k_{Rb}.

Rb uptakes in absence of external K

The amounts of Rb measured in the tracer experiments include the extracellular Rb, since no wash was given before taking the readings. Before plotting these quantities or those found by analysis of muscles which had not been washed a standard deduction of the Rb calculated to be held in an extracellular space of 0·1 ml./g was made. This correction was probably somewhat too small, particularly after long immersions. Analytical results obtained after a 1 min wash in a Rb-free solution were plotted directly. The abscissa against which the uptake was plotted was chosen to be $([Rb]_e t)^{\frac{1}{2}}$. Figure 1 shows that the results obtained over the range of concentrations 2·5–100 m-equiv Rb/l. fall along a single line; the part from 8 to 40 m-equiv Rb/kg tissue is nearly straight. The curve for Rb/K interchange is less steep than the corresponding one for K–K exchange, which is also indicated on the figure. The slope of the Rb-uptake

curve is 0·42 times that of the K-exchange curve. This corresponds to the coefficient k_{Rb} being $(0{\cdot}42)^2 = 0{\cdot}2$ times the coefficient k_K for K exchange.

The effect of temperature on Rb uptake was estimated by comparing the values of the product (concentration × time) required to give a particular

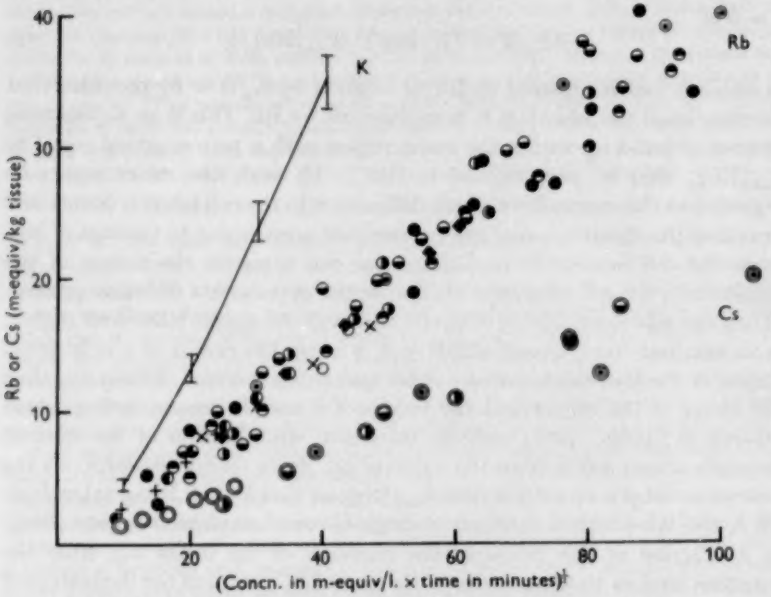

Fig. 1. (K) The line relating K exchanges at 20° C to $([K]_e \times \text{time in min})^{\frac{1}{2}}$ with an indication of the experimental scatter (from Harris & Sjodin, 1961, Fig. 3).

(Rb) The experimental observations described in this paper of the Rb uptakes from various Rb concentrations plotted against $([Rb]_e \times \text{time in min})^{\frac{1}{2}}$. Points for Cl-free mixtures using analytical method; ○ 5, ◑ 10, ◒ 20, ◓ 40, ● 80, ⊙ 100 m-equiv Rb/l. Points obtained in Cl mixtures using tracer method; ◐ 2·5, ◆ 20 m-equiv Rb/l. Points obtained in Cl mixtures using tracer method applied to muscles pre-loaded with ordinary Rb; + 5, × 20 m-equiv Rb/l. Values are corrected when necessary for the Rb in a space equal to 0·1 ml/g.

(Cs) Experimental observations of Cs uptakes from various Cs concentrations plotted against $([Cs]_e \times \text{time in min})^{\frac{1}{2}}$. Points for Cl mixtures using tracer method; ◉ 2·5, ◑ 5 m-equiv Cs/l. Points for Cl-free mixtures (using $CsNO_3$) obtained analytically; ◒ 20, ◎ 100 m-equiv Cs/l.

uptake at 0° C with the value interpolated from the 20° C curve. The ratio so found was 3·4:1 (from 9 readings at 0° C); this according to our model represents the ratio of the value of k_{Rb} at 20° C to that at 0° C. In the K exchange the ratio of the values of k_K at the two temperatures was 3·3:1 (Harris & Sjodin, 1961).

The question arose whether the Rb–Rb exchange might differ in rate from the Rb-K interchange: this was tested by preparing muscles by overnight immersion in a 100 mM-RbCl mixture before following the uptakes of labelled Rb. The muscles contained about 85 m-equiv Rb/kg and 10 m-equiv K/kg. The results of these experiments fall on the same line (the points are distinguished in the figure) showing that the Rb^+–Rb^+ exchange occurs at practically the same rate as the Rb^+–K^+ interchange. In both Rb^+–K^+ and Rb^+–Rb^+ exchanges the postulated outer region becomes occupied by Rb^+ because in each case the solution contains only Rb ions.

The Rb uptake results did not show any regular difference between Cl-containing and Cl-free media. This suggests that there is little associated net movement of Rb^+ with Cl^- into the muscle even from raised RbCl concentrations, since this would have led to the observed uptakes for Cl mixtures exceeding the comparable ones from Cl-free media. The point was tested by comparing the analysis of a muscle soaked for 64 min in

100 mM-RbCl + 90 mM-NaCl + 20 mM-$NaHCO_3$

with that of the paired muscle soaked for the same time in a mixture of

100 mM-$RbHCO_3$ + 20 mM-$NaMeSO_4$.

The total [Rb + K] in the muscle from the Cl mixture was 112 m-equiv/kg, that from the Cl-free mixture was 104 m-equiv/kg, so here only an extra 8 m-equiv Rb/kg was gained. In the comparable case with 100 mM-KCl present net gains of 35–48 m-equiv K/kg tissue were found. That there is some entry of RbCl from mixtures containing high concentrations of the salt can also be shown by following the swelling which occurs when water accompanies the RbCl into the tissue from a mixture having Rb replacing equivalent Na. The time course of swelling of a muscle in 100 mM-RbCl + 20 mM-$NaHCO_3$ + 2 mM-$CaCl_2$ is shown in Fig. 2. A few points from the swelling curve found in a similar mixture but with 100 mM-KCl are indicated (from Harris & Sjodin, 1961, Fig. 5). The results confirm that the water movement and, by inference, the RbCl movement, is slower than when KCl is used. The slope of the line in KCl solution is to that in RbCl solution as 4·5 to 1; to reach the same degree of swelling the muscle in RbCl mixture has to be given $(4{\cdot}5)^2 = 20$ times as long as the one in the KCl mixture. The swelling in a caesium mixture was insignificant in 3 hr. This shows that there is a still greater disparity between the rates of net movement of the respective salts than exists between the rates of the cation exchanges.

Some points for Cs uptakes from solutions having different concentrations are plotted in Fig. 1. Like the Rb results these were obtained alternatively by tracer and analytical methods. The slope of the Cs–K curve is about 0·20 times that of the K exchange curve, which corresponds to k_{Cs} being $(0{\cdot}20)^2 = 0{\cdot}04$ times k_K.

Effect of net Rb gain on Rb uptake

The experiments made in RbCl solutions showed that net gain of the salt was slight. In order to obtain appreciable changes of total content of (K + Rb), use was made of muscles which had first been K-depleted by storage for 48 hr in a K-free solution at 4° C. This procedure follows that

used for obtaining a net K gain and has also been applied by Libin & Schneider (1957) to Rb and Cs uptakes. The point of the present experiments was to find out how the uptake of Rb occurring when the muscle Na content falls compares with the amount of Rb normally exchanging for K. The results obtained after various times of exposure to a mixture containing (in m-equiv/l.) [Rb] = 10, [Na] = 105, [methyl sulphate] = 95,

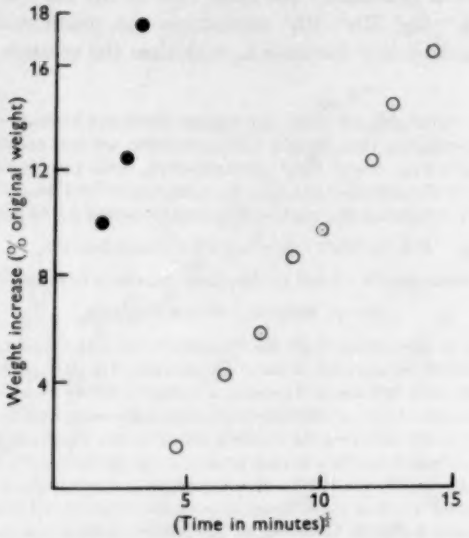

Fig. 2. ○, the swelling of a muscle at 20° C in a mixture having (mM): RbCl 100, $NaHCO_3$ 20, $CaCl_2$ 2. A few points (●) from the corresponding experiment in a KCl mixture are taken from Harris & Sjodin (1961, Fig. 5).

TABLE 1. The contents of Rb, K and Na found in muscles after soaking in a solution at 20° C with 10 m-equiv Rb/l. and 105 m-equiv Na/l., using K-depleted muscles and fresh muscles. A control K-depleted muscle had K = 38·3, Na = 70·4, sum = 108·7 m-equiv/kg. Mean of 5 fresh muscles K = 76·1, Na = 26·4, total 102·5 ± 5·2 (S.D.) m-equiv/kg

1	2	3	4	5	6	7	8	9	10
		Using K-depleted muscles				Using fresh muscles			
		Contents (m-equiv/kg) after 1 min wash in Rb-free soln							
						Means from 3–5 analyses ± S.D.			
Time (min)	$(10t)^{\frac{1}{2}}$	Rb	K	Na	Total	Rb	K	Na	Total
50	22·4	16·4	44·5	45·2	106·1	—	—	—	—
62	24·9	21·3	28·9	59·2	109·4	6·8 ± 0·7	66·5	32·2	105·5
121	34·8	33	36·8	37·7	107·5	10·3 ± 0·7	65·3	29·5	105·1
195	44·4	39·4	18·8	56·3	114·5	15·7 ± 2·3	58·0	27·9	101·6
244	49·5	34·9	42·0	40·8	117·7	18·0 ± 1·7	52·9	32·1	103·0
250	50	34·0	35·3	46·6	115·9	—	—	—	—
328	57·2	42·0	22·5	62·1	126·6	18·8 ± 1·7	55·5	35·0	109·3

$[HCO_3]$ = 20, and 2 mM-Ca acetate are given in Table 1 (col. 3); the Rb uptakes are much greater than usual (col. 7). This shows that there can be a relatively rapid entry of Rb linked with Na extrusion in addition to the Rb–K interchange.

Rb uptakes from mixtures of Rb and K

It is necessary to find out whether the tissue as a whole has any selectivity between K and Rb ions, because this determines the equilibrium levels to which the two ions tend when presented in a mixture. Selectivity within the outer region may be present but its analytical demonstration has not been possible. Lubin & Schneider (1957), using 5 m-equiv/l. of each of Rb and K, found that the Rb content of muscles exposed for 2 days at 25° C was slightly (8 m-equiv/kg cell water) in excess of the K content, while the disproportion in favour of Rb after 3 days was as much as 25 m-equiv/kg water. However, these authors further showed that when loss of K and Rb occurred from a muscle previously loaded with Rb it was the K which tended to escape faster. Since their muscles after 3 days loading contained considerably (35 m-equiv/kg cell water) more Na than at 2 days it is likely that some escape of K and Rb in exchange for Na had taken place. The faster movement of the K would then lead to an increase in the ratio Rb:K within the cell. It seems important to choose conditions, if this be possible, to avoid loss of K or Rb against Na in equilibration experiments. A further disturbing factor would be introduced if chloride were present, on account of the faster rate of KCl movement compared with that of RbCl (as illustrated in Fig. 2). We made our equilibration experiments in media having only non-penetrating anions (HCO_3 and methyl sulphate) present. In making the trials the muscles were frequently transferred to fresh portions of soaking solution to remove the K which had emerged from the cells and to discourage bacterial growth.

The cation contents of muscles which had been exposed for 16–48 hr to various mixtures of K, Rb and Na salts were determined after a preliminary 1 min wash in a cold methyl sulphate solution. The ratio [Rb]:[Rb + K] in the tissue was found to tend towards, but not to attain, the ratio pertaining in the solution (Table 2).

It is possible that the failure to reach equality of Rb:K ratios inside and outside the cell in the mixtures of Rb and K has its explanation in the same phenomenon as is encountered when attempting to exchange muscle K with labelled K. Long exposure of the isolated tissue leads to breakdown of the sodium exclusion. Once Na can enter and displace the internal K, the external ion, be it labelled K or Rb, meets additional competition for entry into the sites of the ion exchanger. Such interference has

been demonstrated using muscles which had been loaded with excess KCl (Harris & Sjodin, 1961, Fig. 9).

We shall proceed on the basis that there is no important difference between the final Rb–K distribution inside and outside the cell although the results in Table 2 only extend to about 85% equilibration. It does not follow that the activity coefficients of K and Rb are equal within the ion exchanger, because the effect of localized concentration differences would not be seen in the gross analysis. A model system in which activity

TABLE 2. The ratio [Rb]:[Rb + K] found for the cell cations after long exposures to media of given compositions at 20° C. The anions present were bicarbonate and methyl sulphate

	Solution composition (m-equiv/l.)			Time of exposure (hr)	Ratio: [Rb]:[Rb+K]		Total Rb+K (m-equiv/kg)
	Rb	K	Na		In soln.	In tissue	In tissue
1	10	10	100	21	0·50	0·39	75·2
2	20	20	80	21	0·50	0·41	87·7
3	20	20	80	27	0·50	0·44	87·0
4	40	40	40	21	0·50	0·43	100·1
5	49	53	23	22	0·48	0·41*	103*
6	50	50	20	18	0·50	0·43	92·3
7	60	55	1·5	21	0·52	0·44*	103·5*
Pre-treated	80	0	33	4	—	—	—
then							
8	49	53	23	17	0·50	0·44†	105†
9	42	11	70	19	0·79	0·66	82
10	42	11	70	26	0·79	0·67	82
11	42	11	70	48	0·79	0·75	100

* mean of 4; † mean of 3.

differences within the ion exchanger do lead to concentration ratios within the *exchanger* differing from those in the external media has been found (Sjodin, unpublished). However in the formulation of the exchange kinetics it is the activities of the ions in the exchanger which are important, they are respectively equal to the products of adsorbed concentration and local activity coefficient. Within the exchanger the respective activities must be proportional to the appropriate applied concentrations, since in solution the activity coefficients of K and Rb are similar; this must in turn lead eventually to the internal Rb:K ratio becoming equal to that outside when *intracellular* activity coefficients of the two ions are similar.

The rate of build up of the adsorbed Rb, in presence of K, can be written

$$\frac{d\mathrm{Rb}_a}{dt} = k_{\mathrm{Rb}}[\mathrm{Rb}]_e(N - \mathrm{Rb}_a) - k_{\mathrm{Rb}}[K]_e\mathrm{Rb}_a, \qquad (3)$$

where the quantities $\mathrm{K}_a = (N - \mathrm{Rb}_a)$ and Rb_a are the activities in the adsorption region. The second term on the right gives the rate of displace-

ment of absorbed Rb by external K. For constant $[Rb]_e$ and $[K]_e$ applied at $t = 0$ one has

$$\frac{Rb_a}{N} = \frac{[Rb]_e}{[Rb]_e+[K]_e}\{1-\exp(-k_{Rb}([Rb]_e+[K]_e)\,t)\}. \qquad (4)$$

Comparing this with the equations (Harris & Sjodin, 1961, eqn. (2); this paper eqn. (2)) for build-up of either labelled K or Rb in absence of the other ion, we see that the final concentration is reduced in the ratio $[Rb]_e:([Rb]_e+[K]_e)$ and that the rate constant for the build-up is

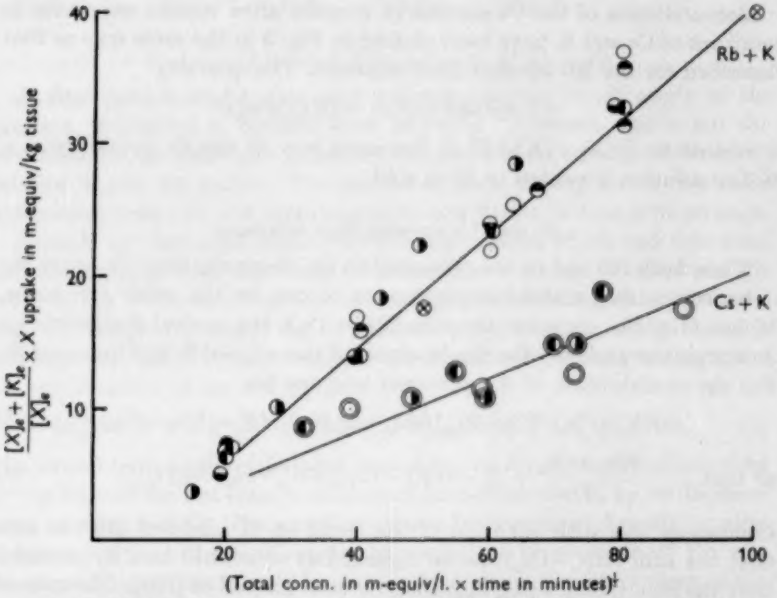

Fig. 3. Uptakes of Rb or Cs at 20° C from mixtures with K. Ordinates are content (m-equiv/kg) × $([X]_e+[K]_e)/[X]_e$ and abscissae are $\{([X]_e+[K]_e)\,t\}^{\frac{1}{2}}$, where X stands for Rb or Cs. Mixtures and methods used: (Rb) (tracer method), ⊗ 2 Rb + 100 K; ◑ 2·5 Rb + 5 K: (analytical method), ◒ 5 Rb + 10 K; ◐ 10 Rb + 10 K; ○ 20 Rb + 5 K; ◓ 40 Rb + 5 K; (Cs) (tracer method), ◎ 10 Cs + 10 K; ◑ 10 Cs + 20 K; (analytical method), ◐ 50 Cs + 50 K. The numbers indicate m-equiv/l.

k_{Rb} $([K]_e+[Rb]_e)$ instead of $k_K[K]_e$ or $k_{Rb}[Rb]_e$. At a given value of $([Rb]_e+[K]_e)\,t$, say V, the Rb content which is observed will then be only $[Rb]_e/([Rb]_e+[K]_e)$ of the content observed in absence of the external K at $[Rb]_e t = V$. To express the Rb uptakes from mixtures in a way consistent with the method used for the ions separately the uptakes are multiplied by $([Rb]_e+[K]_e)/[Rb]_e$ and the products plotted against $\{([K]_e+[Rb]_e)\,t\}^{\frac{1}{2}}$ in-

stead of $([K]_e t)^{\frac{1}{2}}$ or $([Rb]_e t)^{\frac{1}{2}}$ (Fig. 3). For a series of different mixtures of K and Rb this procedure leads to a curve which is the same within experimental variability as the curve obtained for Rb uptakes alone (Fig. 1). The slope of the linear part of the curve is a measure of the square root of the Rb exchange coefficient, $k_{Rb}^{\frac{1}{2}}$. The result can be used in reverse to calculate the Rb content of a muscle which has been exposed to a given K + Rb mixture for a given time.

Cs uptakes from mixtures of Cs and K

Measurements of the Cs content of muscles after various exposures to mixtures of Cs and K have been plotted in Fig. 3 in the same way as that described for the Rb uptakes from mixtures. The quantity

$$\{\text{Cs uptake} \times ([Cs]_e + [K]_e)/[Cs]_e\}$$

is related to $\{([Cs]_e + [K]_e)\, t\}^{\frac{1}{2}}$ in the same way as the Cs uptake from a K-free solution is related to $([Cs]_e \times t)^{\frac{1}{2}}$.

Rb and Cs uptakes from mixtures

When both Rb and Cs are presented to the tissue the two ions enter the outer region and mutual displacements of one by the other can occur. If, however, one considers the sum $(Rb_a + Cs_a)$, the mutual displacements do not matter and only the displacement of the original K is of importance. For the equilibration of the adsorbed ions one has

$$d(Rb_a + Cs_a)/dt = (k_{Rb}[Rb]_e + k_{Cs}[Cs]_e)\,(N - (Rb_a + Cs_a)) \qquad (5)$$

so that

$$\frac{Rb_a + Cs_a}{N} = \{1 - \exp(-(k_{Rb}[Rb]_e + k_{Cs}(Cs]_e)\, t)\}. \qquad (6)$$

Comparing this with equation (2) for build-up of adsorbed Rb one sees that the sum $(Rb_a + Cs_a)$ can be regarded as equivalent to Rb_a provided that the sum $([Rb]_e + (k_{Cs}/k_{Rb})\,[Cs]_e)$ is used instead of $[Rb]_e$. The rate of diffusion of the mixture into the cells can then be predicted from the Rb exchange curve.

In the test of this point shown in Table 3 analyses were made after various exposures to a mixture of 50 m-equiv/l. of both Rb and Cs at 20° C. The usual correction for extracellular ions was applied. Since the uptake curves of Fig. 1 lead to the ratio $k_{Cs}:k_{Rb} = 0{\cdot}04:0{\cdot}2 = 0{\cdot}2$ the mixture should give uptakes equal to those from 60 m-equiv Rb/l., and this is approximately the case. The analyses show that the tissue takes up about 1·5 times as much Rb as Cs. That the disproportion is not as great as that between the exchange coefficients demonstrates the consequence of interaction between the ions.

TABLE 3. Comparisons between observed sums of Rb + Cs uptakes from 50 m-equiv/l. each of Rb and Cs at given times and values predicted from the Rb curve (Fig. 1). 50 m-equiv Cs/l. is taken from the results of Fig. 1 as equivalent to 10 m-equiv Rb/l. The analyses are means of 3 results and have S.D. about ±2 u.

Time (min)	$(60t)^{\frac{1}{2}}$	Cations in tissue (m-equiv/kg)			Value predicted from Rb exchange curve (m-equiv/kg)
		Rb	Cs	Sum	
10	24·5	5·3	2·5	7·8	8·3
25	38·7	8·2	5·7	13·9	14·8
60	60·0	13·7	10·2	23·9	24·0
100	77·4	18·1	9·8	27·9	31·6

Uptakes of labelled K in presence of Rb or Cs

At first sight it might seem that a simple solution would apply to the problem of labelled K uptakes from mixtures. However, this is not the case because there are three components in the system, namely ordinary K, labelled K and Rb (or Cs). The labelled K finds its way into a gradually diminishing reservoir of K and also encounters Rb (or Cs) ions in its passage.

Some K uptakes were measured by using muscles which had first been loaded overnight with Rb. Into these the K movement followed the Rb curve of Fig. 1, showing that the interchange coefficient k_{Rb} determines the rate whichever way round the experiment is performed. Using this one can approach the K uptake from K + Rb mixtures by writing the equation for equilibration of the outer region

$$dK_a^*/dt = k'[K]_e^*(N - K_a^* - Rb_a) - k_{Rb}[Rb]_e K_a^* + k_{Rb}[K]_e^* Rb_a. \qquad (7)$$

The second term on the right is the rate of loss by displacement of adsorbed K* by Rb and the last term is the rate of gain of labelled K by its displacement of adsorbed Rb. The equation cannot be integrated directly because it turns out that the K–K exchange coefficient in presence of Rb is a function of time, presumably because it depends on the Rb content of the outer region. Before proceeding evidence bearing on this will be given.

The course of uptake of labelled K from equal K concentrations with and without Rb present was compared without correction for extracellular ions. Figure 4, curves *A* and *B*, show that K uptake commences similarly from each solution. It is only after about 8 min when the tissue contains some 1·5 m-equiv K/kg that the curves diverge. At this time it can be calculated that the muscle in the Rb mixture contains just over 5 m-equiv Rb/kg. This quantity then gives an indication of how much tissue Rb is necessary for the K movement to be affected. The 1·5 m-equiv K/kg taken up exceeds by a factor of at least two the amount expected for extracellular material and the lag in Rb action cannot be attributed to its being delayed relative to the K in the extracellular space because in water both ions have

the same diffusivities. That the lag is really due to a requirement for a certain accumulation of Rb and is not a spurious effect stemming from extracellular K is further shown by the fact that exposure to Rb before the labelled K causes a perceptible reduction of even the earliest K uptake (Fig. 4, curve *C*).

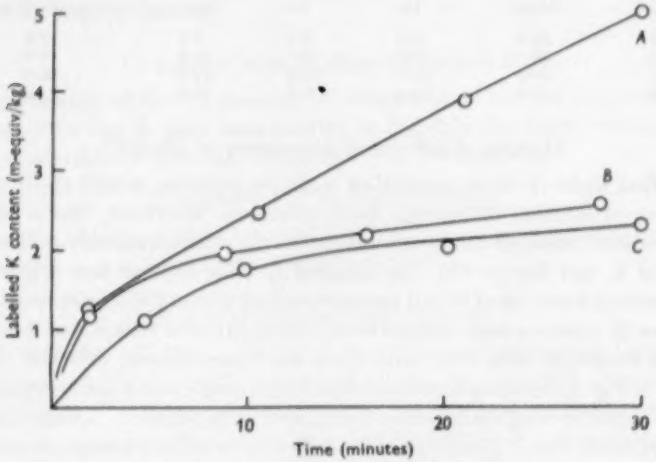

Fig. 4. The early parts of labelled K uptakes at 20° C from (*A*) 5 m-equiv K/l., (*B*) 5 m-equiv K/l. + 40 m-equiv Rb/l. applied at $t = 0$, and (*C*) from the same mixture as (*B*) but applied to a muscle pre-treated with 5 m-equiv K/l. + 40 m-equiv Rb/l. for 75 min before transfer to the labelled K mixture.

It was found that when experiments like that of Fig. 4*B*, in which the ratio of Rb:K applied was 4 or more, were treated in a way corresponding to that described for the Rb uptake from mixtures, by plotting the product of the labelled K content times $([K]_e + [Rb]_e)/[K]_e$ against $\{([K]_e + [Rb]_e)t\}^{\frac{1}{2}}$ the lines ran parallel to, but above, the standard Rb exchange line of Fig. 1. A rapid uptake of K at the beginning of the exposure was responsible for the upward displacement of the line, which took the longer to become parallel to the Rb curve the lower was the Rb concentration applied.

The kinetic results for labelled K uptakes were analysed and shown to fit the hypothesis that the exchange coefficient of K is ultimately reduced to equality to the K–Rb interchange coefficient k_{Rb} as the tissue Rb content increases. This was done in the following way. The actual uptake results for a number of mixtures was plotted (Fig. 5) and curves were computed by rough methods from the data for K and Rb movements already presented with an assumption about the relation between k' and the Rb

content of the tissue. If one integrates eqn. (7) over a short interval, so that k' can be taken as constant, the result is

$$\frac{K_a}{N} = \{1 - \exp(-(k'[K]_e + k_{Rb}[Rb]_e)t)\}$$

$$- \frac{[Rb]_e}{[Rb]_e + [K]_e}\{1 - \exp(-k_{Rb}([K]_e + [Rb]_e)\, t)\}. \qquad (8)$$

This is the difference between two exponential build-ups, so that the consequence of diffusion from this source can be obtained by taking the difference between the respective diffusions from (1st term) a source of K reaching a final concentration N with rate constant $(k'[K]_e + k_{Rb}[Rb]_e)$ and (2nd term) a source of K which reaches final concentration

$$N[Rb]_e/[Rb]_e + [K]_e$$

with rate constant $k_{Rb}([K]_e + [Rb]_e)$. Both quantities can be obtained from the K exchange curves, which are a particular form of transform of the function describing the source concentration. The quantities over the abscissae $\{(k'/k_K)\,[K]_e + (k_{Rb}/k_K)\,[Rb]_e)\,t\}^{\frac{1}{2}}$ correspond to the first term, while those over the abscissae $\{(k_{Rb}/k_K)\,([K]_e + [Rb]_e)\,t\}^{\frac{1}{2}}$ are reduced in the ratio $[Rb]_e/([Rb]_e + [K]_e)$ for the second term. It was arbitrarily chosen to make $k' = 0{\cdot}8\,k_K$ for such time as was necessary for the tissue to gain 6 m-equiv Rb/kg. This time was computed by the method used to plot Fig. 2 and values are listed in the legend of Fig. 5. In the interval between the tissue having 6 and 10 m-equiv Rb/kg the value of k' chosen was $0{\cdot}45\,k_K$, and finally for tissue Rb in excess of 10 m-equiv/kg k' was taken as equal to k_{Rb}.

The rough method of calculation shows that our results are consistent with the idea that the case of K movement is progressively reduced to a final exchangeability equal to that of Rb as the tissue Rb content increases. If one had information about the quantity of Rb actually in the outer region, so that the law relating K exchange coefficient to the local Rb concentration was known, the problem could be applied to a computer.

Uptakes of K in presence of a given Cs concentration are similar to, or slightly higher than those found in presence of comparable concentrations of Rb. Despite its rate of entry being less than Rb, the Cs did not in the times used in our experiments exert a greater hindrance than Rb. This could be because its slower movement keeps the Cs concentration in the outer region at a level proportionately less than the Rb level. Two sets of the experimental points found in K + Cs mixtures have been shown on Fig. 5.

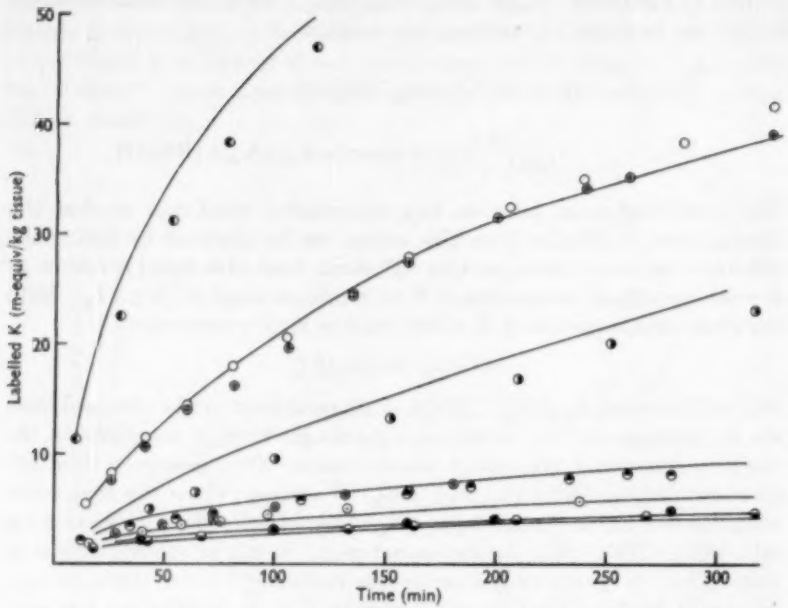

Fig. 5. Uptakes of labelled K at 20° C from mixtures with Rb or Cs corrected for the amount held in extracellular space equivalent to 0·1 ml./kg. The curves drawn have been computed according to the method described in the text which takes account of a reduction of the rate coefficient of K exchange as the tissue Rb content increases. The mean value of the coefficient, relative to that holding in Rb-free solution, was taken as 0·8 for Rb contents between 0 and 6 m-equiv/kg, 0·45 for Rb contents between 6 and 10 m-equiv/kg and 0·2 (the same as the Rb rate coefficient) for Rb contents exceeding 10 m-equiv/kg. The times at which the tissue Rb contents reached 6 and 10 m-equiv/kg were calculated by using in reverse the method applied for plotting Fig. 2.

Mixtures			Time (min) for Rb content to be:	
K	Rb (m-equiv/l.)	Symbol	6 m-equiv/kg	10 m-equiv/kg
46	21	◐	39	91
10	5	○	146	365
5	2·5	◑	291	730
5	20	◓	22	47
5	40	⊙	10	22
2·5	25	●	15	35
5	80	◒	5	11
	Cs			
10	5	⊕		
5	25	⊗		

No attempt to fit the results in the Cs mixtures was made, but it appears that a similar procedure would be applicable.

DISCUSSION

The nature of the results obtained has required their discussion in the body of this paper so here it is appropriate to refer to a few salient features. It can be said to be well known that K, Rb and Cs fall in a diminishing series of ease of penetration into muscle, and the figures derived from the relative exchange coefficients obtained by squaring the slopes of the linear parts of the curves in Fig. 1 are 1:0·2:0·04. It is suggestive that Rb and Cs not only compete with K but also reduce its exchangeability (as deduced earlier by Sjodin, 1959). Some such interaction, due in his view to a distortion of pore dimensions, has been proposed by Mullins (1956) to explain the action of ions which affect cell permeability. Our explanation of the effect rests rather on the greater adsorption of Rb and Cs on fixed sites along the channels through which the K must pass.

The model we have consistently used in presenting the K, Rb and Cs data requires that the cells 'outer region', which may be the membrane and reticular complex, should have a cation-exchange property holding not more than 10 m-equiv of K or K-like ion/kg tissue. Although various authors (e.g. Ling, 1960) have used competitive adsorption as the basis for interpretation of the interaction between cations entering muscle (not to mention the many papers concerned with other kinds of cell), our present approach extends this by attributing as much importance to the rate of equilibration of the ion-exchange region as to the ionic composition ultimately attained there.

In the results for exchange of muscle K, whether with labelled K (Harris & Sjodin, 1961) or with Rb, there has been no indication that the external Na concentration affects the process. On the other hand, the movement of the Na of high-Na muscles has been shown to be influenced by the external K concentration (Keynes, 1954; Edwards & Harris, 1957). That Na movement from high-Na muscle has an ion-exchange character may be inferred from the observation (Harris, 1950) that Na output to glucose solution is much slower than to an ion-containing medium. Keynes & Swan (1959), using media containing Li ions, have concluded that at least half the Na movement was an ion exchange. May we then regard the outer region proposed in our theory as accommodating both Na and K ions but in separate channels or on different sites? Since K movement is so insensitive to Na, it appears that the latter does not compete for the K sites, while the influence of K on Na movement, and the possibility of displacing muscle Na by K (Carey & Conway, 1954, Table 1) shows that K can occupy Na sites. It seems possible that the slowing of K movement by Rb ions may have the same mechanism as the acceleration of Na movement by K ions. In each case a group or line of ions may be involved and an essential inter-

change movement has a higher probability when a K ion is on a particular site than when either Rb or Na is present. That in some cells Na exchange may, in some instances, follow the same law as K and Rb exchanges follow in muscle, can be inferred from the curves for human erythrocytes given by Harris (1960, Figs. 5, 6). The slopes of the uptake curves plotted against $(\text{time})^{\frac{1}{2}}$ are roughly in proportion to the square root of [Na] applied.

The reduction that we find of the K exchangeability to the K–Rb value as the muscle Rb increases suggests that the adsorption sites in the outer region are present in sufficient depth to ensure that when 10 m-equiv Rb/kg has been reached there is at least one Rb ion in each pathway for K exchange.

It is notable that no conditions were found in the present K + Rb experiments in which the labelled K content would rise through a maximum and fall subsequently as Rb entered. From the beginning the uptake from a mixture is constrained to proceed towards its eventual equilibrium level with equal (or nearly equal) internal and external Rb:K ratios. This is itself evidence that the Rb:K ratio in the outer region is established early in the process and thereafter controls the ratio of Rb to K entering the tissue.

The impediment of K movement by Rb and Cs should have an effect on the electrical properties of the tissue. Since normally the Cl ion carries some $\frac{2}{3}$ of the current (Hodgkin & Horowicz, 1959; Hutter & Noble, 1960) any change of the cation component of conductance will best be seen in absence of Cl (or other penetrating anions). A qualitative experiment in which the internal–external resistance of few fibres from each of a pair of muscles in solution containing respectively 3 mM-K or Rb with Na methyl sulphate showed that the values in the Rb solution were 2–4 times those in the K solution. Experiments now in progress indicate that the Cl exchange process follows the same pattern as that we have described for the cations.

SUMMARY

1. The uptakes of Rb and Cs by frog sartorius muscles have been measured and the interaction between K, Rb and Cs has been studied.

2. The uptakes of Rb from various mixtures of Rb and Na salts and the uptakes of Cs from various mixtures of Cs and Na salts are made to fall along single curves for a given temperature by the expedient of plotting them against a function of (concentration × time of exposure).

3. By choosing the square root of (concentration × time) as abscissa the respective uptake curves for Rb and Cs can be compared with that previously obtained for K exchange.

4. The results are interpreted, using as model an interior compartment

fed from an outer region having ion-exchange properties. The relative exchangeabilities of K for K, Rb and Cs respectively are as 1:0·2:0·04.

5. Uptakes of Rb or Cs from mixtures with K or with each other are shown to be consistent with the predictions from the model. The results can be fitted to the curves obtained for the single ions.

6. Entry of Rb into previously K-depleted muscles so that Na is displaced by Rb is faster than the interchange of Rb with the muscle K.

7. Uptake of labelled K from mixtures with Rb or Cs follows a course consistent with the hypothesis that when Rb or Cs has entered the ion-exchange region the K exchange is slowed down. In the case of Rb + K mixtures the K exchange ultimately falls to the same rate as holds for Rb–K interchange, but Cs takes longer to exert its full inhibitory effect.

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THE EFFECT OF AGE ON THE FORMATION OF HISTAMINE IN THE RAT

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During the last 3 days of pregnancy the rat foetus produces histamine at a high rate, about 80% being formed in the liver (Kahlson, Rosengren & White, 1960). As the histamine-binding capacity of the foetal tissues is low, histamine diffuses freely into the surrounding fluid and is excreted by the mother. The maternal excretion of urinary histamine rises from about the 15th day of pregnancy, climbs to a peak in the last 1–2 days before term, and then falls abruptly (Kahlson, Rosengren & Westling, 1958; West, 1960).

We have studied in detail the histidine decarboxylase activity both of the rat foetus from the 13th day of pregnancy until term, and of rat tissues in early life. A preliminary communication of the results has been given to the Physiological Society (Telford & West, 1961*a*).

METHODS

Rats of Wistar strain weighing 150–250 g before pregnancy were used. They were fed on a cube diet (No. 41B, Associated London Flour Millers, Ltd.), allowed drinking water *ad lib.* and housed at 70 ± 1° F (21° C).

Each day from the 13th day after mating until term three or more rats were killed by decapitation, and the foetuses removed and pooled. Livers were dissected from some of the foetuses. The histidine decarboxylase activity and the histamine content of the foetus, foetal liver and foetus minus liver were then determined. On occasion, the stomach, intestines, lungs and kidneys were dissected from some of the foetuses and treated similarly. The enzyme activity and histamine content of tissues from new-born and maternal rats were also estimated.

Measurement of histamine formation in vitro. The method of Waton (1956), slightly modified as described in detail by Telford & West (1961*b*), was used. Briefly, pooled tissue from freshly killed rats or foetuses was cleaned and weighed, and ground in a glass mortar with a little sand and Tyrode solution (5 ml./g tissue). The resulting homogenate was allowed to stand and the supernatant fluid was removed for incubation. The composition of the incubation mixture was as follows:

Tissue homogenate (400 mg)	2·0 ml.
L-histidine (neutralized, 15 mg/ml.)	0·5 ml.
Aminoguanidine (neutralized, 10 mg/ml.)	0·05 ml.
Benzene	20 mg
Phosphate buffer (0·05 M-KH_2PO_4)	2·45 ml.

Histidine, the substrate, was always added last. Aminoguanidine was added to inhibit histaminase. The mixture was immediately shaken and incubated for 3 hr at 37° C. The reaction was stopped by reducing the pH of the solution to 4·0 with N-HCl and by cooling to 4° C. After the mixture had been neutralized with N-NaOH, its histamine content was determined. In each experiment mixtures with and without the substrate and mixtures containing boiled homogenate or no homogenate were incubated and assayed for histamine. Each incubation was performed in duplicate. The final volume of the mixtures was 5 ml. The mean histamine content of the mixtures incubated in the presence of histidine less the mean histamine content of mixtures incubated in the absence of histidine gave the amount of histamine formed from histidine. The amounts of histamine formed per gram of tissue and per tissue were used as indices of histidine decarboxylase activity. Each result is the mean of at least two experiments.

Extraction of tissues for histamine. Each tissue was cleaned and weighed, cut into small pieces and extracted with 10% (w/v) trichloroacetic acid, 5 ml./g tissue, for 24 hr. Excess acid was removed by shaking the supernatant with 4 vol. of anaesthetic ether four times, the ethereal layers being discarded. After gentle heating to remove dissolved ether the aqueous residue was assayed for its histamine content. Each result is the mean of at least two experiments.

Assay of histamine. Bioassays were performed on the isolated ileum of the guinea-pig. A 15 ml. bath of oxygenated atropinized Tyrode solution at 32° C was used. On occasion, extracts were also assayed on the blood pressure of an anaesthetized cat. The specificity of the responses was checked with mepyramine maleate. All values of histamine refer to the base.

RESULTS

Factors affecting histamine formation by rat foetal tissues

The histidine decarboxylase activity of foetal tissues was tested at various pH values, as it had been found in a previous study (Telford & West, 1961*b*) that the optimal pH value for this enzyme in the adult rat varies from tissue to tissue; in adult liver, for example, it is 8·0 whereas in pyloric stomach it is 7·0. The results of the present experiments show that for foetal tissues at all stages of pregnancy the optimal pH is 6·5. This is shown in Fig. 1, where the rates of formation of histamine by foetal and adult livers are compared at various pH values.

The presence of benzene is essential for the detection of enzyme activity in the liver of the adult rat, but benzene does not increase the activity of the foetal liver (Table 1). Pyridoxal, which is considered to be a co-enzyme of mammalian histidine decarboxylase (Blaschko, 1957; Rothschild & Schayer, 1958; Kahlson & Rosengren, 1959), also does not increase the activity even when amounts of up to 1 mg are added to the incubation mixture.

Histamine formation in the rat foetus

The histidine decarboxylase activity of the foetus and the foetal liver on each day of gestation from the 13th day until term is shown in Table 2. When calculated per gram of tissue, the activity increases rapidly to reach a peak on the 14th to 15th day of gestation and continues at a high rate

throughout the remainder of pregnancy. When calculated per tissue, however, the peak occurs at birth after which there is a dramatic fall almost to zero (see Fig. 2 and Table 3); the values for foetal liver and foetus are similar and indicate that the liver is the major source of histamine production in the foetus. Furthermore, histidine decarboxylase activity has not been detected in the foetal stomach, intestines, lungs or kidney.

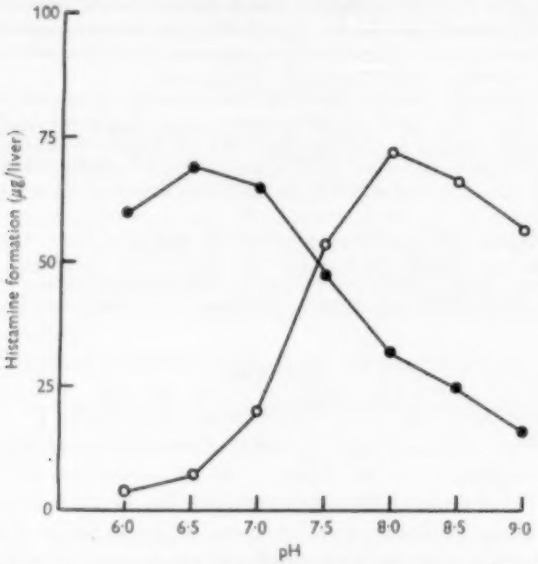

Fig. 1. Effect of pH on the rate of histamine formation in 3 hr in the liver of foetal (●—●) and adult (○—○) rats. Foetuses used at 20th day of gestation.

TABLE 1. Effect of benzene on the rate of histamine formation in the liver of foetal and adult rats. Incubation for 3 hr at optimal pH

	Incubation pH	Histamine formed (μg/tissue)	
		Presence of benzene	Absence of benzene
Adult liver	8·0	72·1	1·7
Foetal liver	6·5	65·4	66·8

Histamine formation in the rat during early life

The histidine decarboxylase activity of the liver of rats killed at varying times after birth was investigated to determine more precisely when the decrease in enzyme activity occurred. The results are plotted in Fig. 3 and indicate that the enzyme activity, when calculated per tissue, decreases rapidly a few hours after birth and is almost zero by 48 hr. After 4 days

TABLE 2. Effect of age on the rate of histamine formation in tissues of rat foetuses. Incubation for 3 hr at pH 6·5

Day of pregnancy	Foetus		Foetal liver	
	μg/g	μg/foetus	μg/g	μg/liver
13	10·0	1·4	1800·0	3·6
14	13·6	4·4	2649·6	13·3
15	32·7	13·8	1867·6	18·7
16	32·6	17·6	931·8	16·8
17	26·8	24·6	529·4	18·5
18	18·7	30·4	458·5	22·9
19	8·2	37·8	473·8	37·9
20	7·9	55·2	436·0	60·0
21	8·0	68·8	446·9	84·9

TABLE 3. Effect of age on the rate of histamine formation in tissues of young rats. Incubation for 3 hr at pH 6·5

Day after birth	Young rat		Liver	
	μg/g	μg/rat	μg/g	μg/liver
1	3·1	29·0	95·0	20·9
2	0	0	1·1	0·3
3	0	0	2·0	0·6
4	0·2	1·4	2·2	0·7

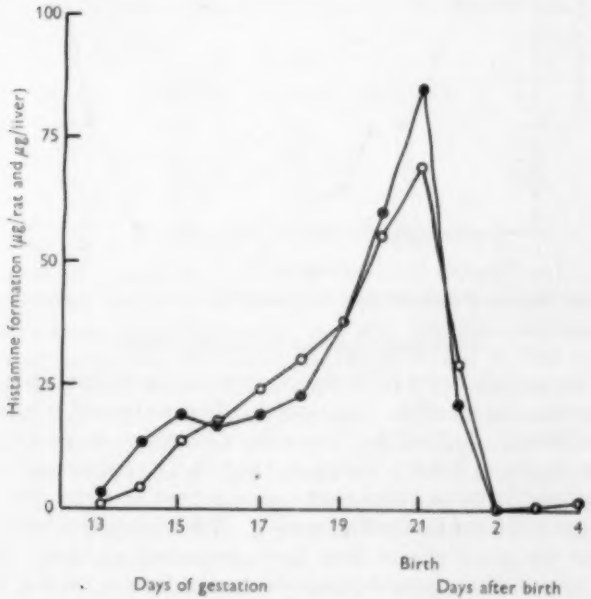

Fig. 2. Effect of age on the rate of histamine formation in 3 hr in rat foetuses and young rats (○—○) and in the liver of rat foetuses and young rats (●—●).

activity returns but only when incubation proceeds at an alkaline pH and in the presence of benzene, adult values (72 μg/tissue) being reached after about 100 days. When the enzyme activity is calculated as histamine formed per gram of tissue, the adult level (10 μg/g) is reached at weaning time (about 21 days).

Histidine decarboxylase activity has not been detected in the stomach, intestines, lungs or kidneys of young rats up to 10 days. Thereafter, activity is found in the pyloric stomach and adult values (9 μg/tissue or 18 μg/g) are reached by about 30 days.

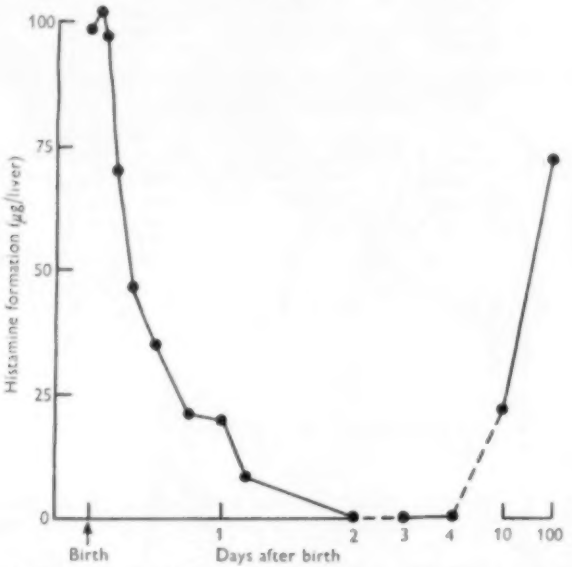

Fig. 3. The amount of histamine formed in 3 hr in the liver of young rats.

Histamine content of the rat foetus

Many discrepancies exist in the figures reported for the histamine content of various foetal rat tissues. We have therefore examined litters on each day of pregnancy, pooling the tissues from members of each litter. The results are shown in Table 4 (columns 3 and 5). The histamine contents of both the foetus and the foetal liver rise to a peak at about the 17th day of gestation and then markedly decrease. The histamine contents of the foetal liver are much higher, than those previously reported. Up to the 18th day of gestation the *total* histamine in the liver is similar to that in the foetus (Table 4, columns 6 and 7), but thereafter the histamine in the foetus continues to rise whilst that in the liver does not increase.

Histamine content of the rat during early life

After birth the histamine content of the young rat continues to rise but there is an abrupt fall in liver histamine during the first 48 hr after birth (Table 5, columns 6 and 7). This fall in liver histamine occurs at the same time as the dramatic decrease in the histamine-forming capacity of this tissue.

TABLE 4. Histamine content (μg/g) of tissues of rat foetuses. Estimates made after extraction with Tyrode solution or trichloroacetic acid

Day of pregnancy	Foetus		Foetal liver		Histamine (μg)	
	Tyrode	Trichloroacetic acid	Tyrode	Trichloroacetic acid	Foetus	Foetal liver
15	3·8	2·5	50	60	0·5	0·6
16	5·0	6·2	68	50	1·5	1·1
17	3·5	4·0	70	92	2·5	2·6
18	4·0	4·5	47	66	3·3	3·3
19	5·2	4·1	40	38	6·0	3·0
20	4·1	3·6	32	21	9·1	3·1
21	6·2	3·1	28	16	12·0	3·3

TABLE 5. Histamine content (μg/g) of tissues of young rats. Estimates made after extraction with Tyrode solution or trichloroacetic acid

Day after birth	Young rat		Liver		Histamine (μg)	
	Tyrode	Trichloroacetic acid	Tyrode	Trichloroacetic acid	Young rat	Liver
1	4·6	2·2	11	12	10·2	2·6
2	3·8	2·0	3	3	11·4	0·8
3	2·0	2·9	2	3	17·4	0·8
4	2·4	3·2	1	2	22·4	0·7

Binding of newly-formed histamine

Estimates of histamine in homogenates of tissues made in Tyrode solution give the amounts of histamine not bound to tissue components, while those made in trichloroacetic acid give the total extractable histamine. These estimates are shown in Tables 4 and 5. The amounts of histamine extracted with Tyrode solution are in most cases similar to those extracted with trichloroacetic acid. This indicates, as Kahlson *et al.* (1960) have pointed out for a few foetal tissues, that the histamine formed in the foetus easily diffuses into the surrounding fluid and only a small proportion is retained by the tissues. Similarly, histamine is freely diffusible in the young rat during the first 4 days after birth. By contrast, in similar experiments with the skin of the adult female rat, only about one half of the tissue histamine is extracted with Tyrode solution.

DISCUSSION

Kahlson and his colleagues (Kahlson *et al.* 1960) examined rat foetuses on the 19th, 20th and 21st days of gestation and found a high histidine decarboxylase activity in all tissues and particularly in the liver. These authors suggested that this activity is linked with the general processes of growth. The present study was initiated to test this hypothesis by estimating the enzyme activity both in the rat foetus throughout pregnancy and in the young rat.

In the first place, the results show that the rat foetus is endowed with considerable histamine-forming capacity throughout the last 7 days of pregnancy. The enzyme activity, however, may be calculated in two ways. When calculated per unit weight (e.g. per gram) of tissue, histidine decarboxylase activity reaches its peak by the 15th day of gestation; it then declines slowly until birth and falls to extremely low values during the first 2 days of life. On the other hand, when calculated as the amount of histamine formed per tissue, the histamine-forming capacity is found to rise steadily throughout the latter third of pregnancy to reach its peak shortly after birth. As the weights of the foetal tissues are rapidly increasing throughout pregnancy and are always less than 1 g, calculation of enzyme activity per tissue gives a more physiological estimate of their histamine-forming capacity.

Secondly, the results confirm that the liver is the major source of histidine decarboxylase in the rat foetus. As the foetal liver at no time contains more than a few micrograms of histamine, the amine may be formed by the liver to function elsewhere in the body. This hypothesis is supported by the findings that the histamine in the foetus is not bound to tissues and is easily extractable with Tyrode solution. During the latter third of pregnancy the newly-formed histamine is rapidly transferred to the mother and excreted. The increase in the histidine decarboxylase activity of the foetal liver begins at about the 14th day of pregnancy and results in an increase in the maternal excretion of histamine. Both the histamine formation by the foetus and the histamine excretion by the mother reach a peak about term and then fall rapidly. The histamine excretion by the mother has already been shown to be directly related to the number of foetuses in the litter (Kahlson *et al.* 1958).

The results also illustrate that the optimal conditions for determining the histidine decarboxylase activity in rat liver vary with the age of the rat. The enzyme in foetal liver, for example, requires an acid medium, whereas that in the adult liver requires an alkaline medium. Organic solvents such as benzene enhance the enzyme activity in the adult but

have no effect on that in the foetus. Thus, in the rat, there may be more than one enzyme capable of decarboxylating histidine.

The histamine-forming capacity of the foetal liver, as determined in the present work, is many times higher than that reported by Kahlson *et al.* (1960), who used a tracer technique involving a smaller quantity of substrate. However, when calculated per tissue it is at no time much greater than that of the adult liver, considered by Telford & West (1961) to be the most potent source of histidine decarboxylase in the rat. Up to the 18th day of gestation it is considerably less than the adult value and only during the last 2 days of pregnancy is the adult value approached. Enzyme activity is undetectable in the foetal liver before the 13th day of gestation, and little activity is found in the liver of the young rat: in both cases, rapid growth is occurring. It seems, therefore, that histamine formation is not related to the process of growth. Furthermore, in the adult rapid growth has ceased, but the rate of histamine formation is high.

Little is known concerning the physiological function of histamine formation in the rat. The foetus is capable of forming histamine at a fast rate and this readily diffuses into the maternal tissues. Histamine is an inhibitor of uterine movement in the rat, so the foetus may be producing the amine to help maintain a relatively quiescent uterus until term. The significance of the high rate of histamine formation in the rat foetus may become clearer when it is known how the process is initiated and what is responsible for its rapid decline just after birth. It would be interesting also to know the stimulus for the return of histidine decarboxylase activity in the young rat.

SUMMARY

1. Histamine formation in the rat foetus begins about the 13th day of gestation and is very high during the last 7 days of pregnancy. At birth a dramatic fall in histamine formation occurs, and little activity is detected 48 hr later.

2. The site of histamine formation in the rat foetus is the liver. Other foetal tissues do not form histamine.

3. The optimal pH for the estimation *in vitro* of histidine decarboxylase in the liver of the rat foetus is 6·5. About 4 days after birth the pH for optimal activity changes to 8·0, and traces of benzene are also needed.

4. Up to the 18th day of gestation the liver contains most of the foetal histamine. Thereafter the histamine in the foetus increases but that in the liver does not. After birth the histamine in the liver rapidly decreases to a low level.

5. The histamine-binding capacity of the tissues of rat foetuses and young rats is feeble, Tyrode solution extracting as much histamine as trichloroacetic acid.

6. The histamine-forming capacity of the rat foetus is less than that of the liver of the adult rat, except on the last 2 days of pregnancy.

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CHANGES IN CONDUCTION VELOCITY AND FIBRE SIZE PROXIMAL TO PERIPHERAL NERVE LESIONS

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Our interest in the changes that occur proximal to peripheral nerve lesions was stimulated by the finding that conduction velocity may be reduced proximal to such lesions in man. This has been shown for compression of the deep branch of the ulnar nerve in the hand (Ebeling, Gilliatt & Thomas, 1960), compression of the median nerve at the wrist (Thomas, 1960) and after suture of the median and ulnar nerves at the wrist following traumatic section (Gilliatt, 1961).

Previous investigations in animals have yielded conflicting results. There have been a number of reports that conduction velocity in the central stump is reduced after peripheral nerve section. Acheson, Lee & Morrison (1942) severed the phrenic nerve distally in cats and found that conduction velocity was usually slower in the affected nerve than in the intact nerve after 1–3 weeks. Similarly, Gutmann & Holubář (1951) reported that conduction velocity becomes progressively reduced in the central stump after section of the peroneal nerve in the rabbit. More recently Eccles, Krnjević & Miledi (1959) and Kiraly & Krnjević (1959) showed that in various limb nerves of the cat conduction velocity was slowed proximal to sections performed 6–39 days previously.

On the other hand, Sanders & Whitteridge (1946) found that when the peroneal nerve of the rabbit was crushed, conduction velocity was slightly *increased* proximal to the lesion between 56 and 123 days after operation, and normal at later stages (456 and 486 days). Furthermore, Kiraly & Krnjević (1959) reported that 40 days after the nerve to the lateral head of gastrocnemius of a cat had been crushed conduction velocity was only slightly less than normal.

In an attempt to clarify the issue we have made observations on the peroneal nerve of the rabbit after a variety of lesions and have followed the changes in conduction velocity and nerve-fibre diameter occurring proximal to the lesion between 7 and 446 days after operation. It seemed possible that the disparity between the results of previous investigators might be explained in terms of the different lesions inflicted and the varying

times after operation at which the observations had been made. Apart from the types of lesion made by previous workers, namely crushing and section, we have also examined the effects of section and immediate suture and those produced by applying a persistent constriction to the nerve. Gutmann & Sanders (1943) found that fibres in the central stump above a crush show a reduction in diameter for at least 130 days, but that at later stages (200–300 days) fibre size returns to normal. After severance and suture, although a similar initial diminution occurred, the subsequent increase was not seen, even as late as 346 days after suture. It therefore seemed possible that simple crushing might affect conduction velocity proximal to the lesion differently from severance and suture. Furthermore, different effects might also be produced by a persistent partial constriction applied to the nerve. Weiss & Hiscoe (1948) have shown that under such circumstances axoplasm accumulates in the fibres above the lesion because of interference with its normal proximal-distal flow.

METHODS

The experiments were all performed on adult rabbits which were unselected with respect to sex, weight and breed. At a preliminary operation, carried out under sodium pentobarbitone (Nembutal, Abbott Laboratories, Ltd.) anaesthesia with full aseptic precautions, the lesions were made on the left peroneal nerve in the lower part of the thigh approximately 0·5 cm above the point at which it passes beneath the lateral head of gastrocnemius. The opposite nerve was left untouched and used for control observations.

Four types of lesion were made: simple crushing, section with avulsion of the peripheral stump, section and immediate suture, and ligature. The majority of the observations were made with simple crush lesions (fourteen animals), in which the nerve was crushed with smooth-tipped forceps for 10 sec. With this procedure degeneration of all myelinated fibres distal to the lesion occurs, but since continuity of the endoneurial tubes is preserved regeneration is rapid and complete (Gutmann, Guttmann, Medawar & Young, 1942). In eight animals the nerve was cut with scissors and the peripheral stump avulsed so that regeneration failed to occur and a neuroma formed at the tip of the central stump. In six animals the nerve was cut and the ends united with a fibrin clot (Young & Medawar, 1940); under these circumstances, regeneration is less complete than after crushing, recovery of both fibre number and size in the peripheral stump being defective (Gutmann & Sanders, 1943). Finally, in three animals a steel wire ligature was tied around the nerve so as partially to constrict it and left *in situ*. This produces an initial crush lesion with degeneration of all the larger myelinated fibres distal to the lesion, only a few small myelinated fibres remaining intact. Under these circumstances regeneration occurs, but fibre size distal to the ligature remains small (Weiss & Taylor, 1944; Duncan, 1948).

Conduction velocity was measured *in vivo* under urethane anaesthesia (2·5 g/kg), after survival periods of 7–446 days. The peroneal nerve was carefully exposed and separated from the tibial trunk from the sciatic notch to the knee, and large skin flaps were raised to form the walls of a bath which was filled with liquid paraffin. The temperature of the rabbit was maintained by an electric blanket and warm liquid paraffin was added to the bath as necessary before each measurement of latency to keep the temperature measured beside the nerve trunk within 0·5° C of the initial temperature. The latter was within the range 36–38° C and was made the same for both sides of each animal.

A pair of silver-wire electrodes with a separation of 3 mm was placed on the distal end of the nerve just above the level of the lesion for stimulation. The proximal electrode of the pair delivered negative stimulus pulses of about 0·1 msec duration derived from an isolating transformer, and the amplitude of the pulse was set to elicit a maximal A fibre action potential at the first latency measurement and not altered subsequently during the experiment. The recording electrodes were placed at distances of 10–70 mm proximal to the stimulating electrodes and consisted of another pair of silver wires with a separation of 5 mm, connected through cathode followers to an amplifier with a high rejection ratio against in-phase signals. The clamps supporting the skin round the paraffin bath were earthed.

After each measurement of latency a fine silk thread was tied firmly around the nerve trunk at the level of the more distal recording electrode, and the latter moved distally for the next measurement. At the end of the experiment the position of the stimulating cathode was similarly marked upon the nerve trunk, and the distances from the point of stimulation to each recording point were determined *in situ* with dividers after killing the animal with an overdose of Nembutal.

Each oscilloscope trace contained a 10 kc/sec marker locked both to the time base and to the stimulator. The traces were photographed directly on paper without reduction. The latencies of three points were measured: the first point of inflexion of the A fibre action potential where a tangent drawn down the rising face of the deflexion met the base line, the point half way up the rising face, and the highest point above the base line. Since the stimulus was rigidly locked to the time base of the oscilloscope, the measurements of latency were made from the first time marker, as this was more precisely definable than the onset of the stimulus artifact. The latencies were plotted against conduction distances and a straight line drawn by eye as nearly as possible through the points, not including the origin. The velocity determined from the slope of this line was found to be within 1 % of the velocity calculated by linear regression which was performed whenever the normal and operated nerves showed closely similar velocities.

After measurement of conduction velocity, specimens of the peroneal nerve on both sides were taken for histological examination from the distal, middle and proximal portions of the length of nerve examined. The distal specimen was 1–2 cm above the level of the lesion and the proximal usually 6–7 cm above. Except in the neuroma experiments a specimen was also taken 1 cm below the lesion. The segments of nerve removed were attached to card frames and fixed in Flemming's solution, embedded in paraffin wax and 5 μ transverse sections stained by the modified Weigert method described by Gutmann & Sanders (1943). In one animal the fixation was not adequate for satisfactory measurements to be made.

Since conduction velocity was measured for the fastest fibres in the nerve trunk, the anatomical observations were confined to the largest fibres. The mean values for the outside diameter of the myelin sheath (total fibre diameter) and that for the inside diameter of the sheath (axon diameter) for the five largest fibres in each of four sections at each level were obtained by direct measurement at a magnification of $\times 1000$ using an ocular micrometer. Except where specified, the means of the values obtained from the three levels on each side have been utilized, each mean thus being derived from 60 measurements. A more extensive examination was not technically feasible in a nerve of this size, but the changes in fibre diameter are being further studied by a more detailed analysis performed on the nerve to the medial head of gastrocnemius (J. T. Aitken and P. K. Thomas, unpublished). In some nerves longitudinal sections were also taken for examination of the nodes of Ranvier.

The results on the operated nerves, both for conduction velocity and fibre diameter, have been expressed as percentages of those from the unoperated side. A possible objection to the use of the unoperated nerve for control purposes is the report by Greenman (1913) that fibre diameter is reduced in the corresponding nerve of the opposite side after crush lesions of the peroneal nerve of the rat. The validity of this finding, however, has been questioned (Quilliam, 1958).

RESULTS

Changes in conduction velocity

When the three measurements of the latency of each action potential were compared, the latency of the point half way up the rising face of the spike was found to be more accurately proportional to conduction distance than the point of inflexion or the peak, which were less easily defined. Only results based on half-rise latencies will therefore be described,

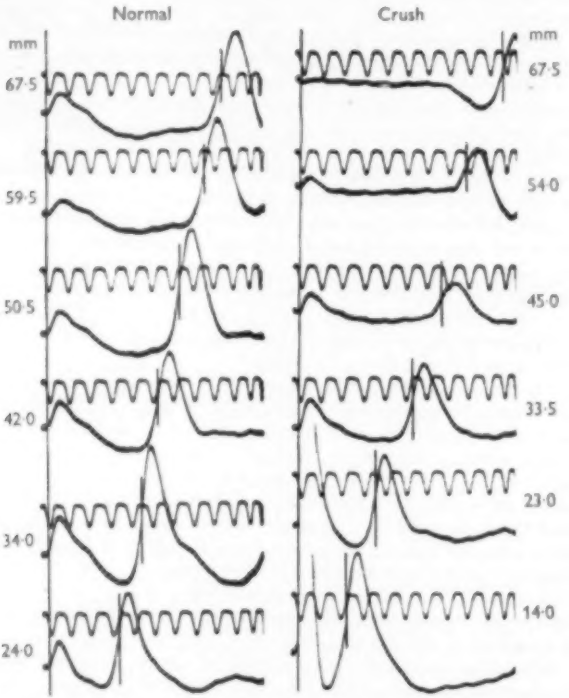

Fig. 1. Action potentials in a normal peroneal nerve and in the contralateral nerve crushed distally 140 days previously. Time marker, 10 kc/sec; conduction distances in mm beside the traces. Five superimposed traces at a repetition rate of 1/sec were recorded in each photograph.

although, whenever the comparison of the velocities of the normal and damaged nerves was critical, it was verified that the other methods of measuring latency gave the same qualitative result. An example of the records obtained is shown in Fig. 1, where each photograph contains 5 superimposed traces due to stimuli delivered at intervals of 1 sec. The

positions of the onset of the stimulus artifact and the half rise point have been indicated, but the latencies plotted in Fig. 2 were measured from an arbitrary zero which was the first peak of the marker on the trace (see Methods). The slope of the lines in Fig. 2 indicates a velocity of 84·9 m/sec for the normal nerve and 70·3 m/sec for the crushed nerve, velocity in the latter being 83 % of normal. The results will be expressed as a proportional reduction in this way since in the thirty-three rabbits investigated, velocities in the normal peroneal nerves ranging from 44 to

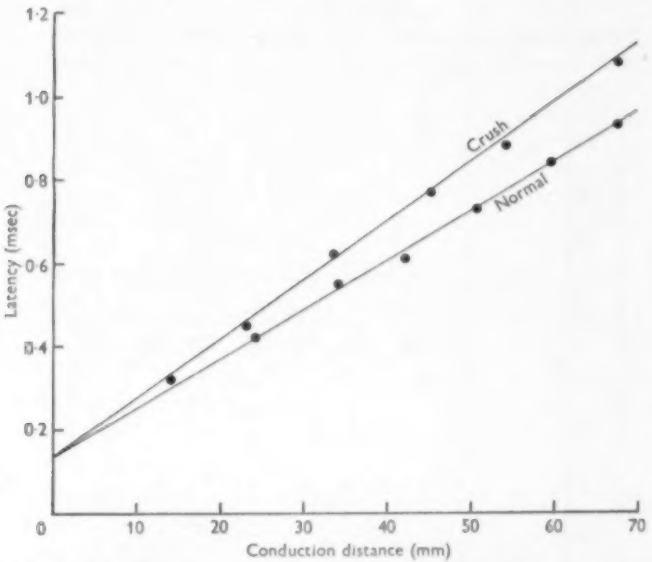

Fig. 2. The latencies of the records shown in Fig. 1 plotted against conduction distance. The normal nerve had a conduction velocity of 84·9 m/sec, the crushed nerve 70·3 m/sec.

108 m/sec were encountered. In order to obtain some assessment of the natural variation between the two sides in a single animal and also the accuracy of the measuring procedure, the conduction velocities of the peroneal nerves were measured on both sides in two normal rabbits and found to be 92·9 and 95·8 m/sec, differing by 3·1 % in one rabbit, and 81·4 and 83·7 m/sec, differing by 2·8 % in the other.

Measurements of conduction velocity in the peroneal nerves of thirty-one rabbits with distal nerve injuries are plotted in Fig. 3. After crush, constriction, or section and suture injuries the conduction velocity above the lesion falls to less than 90 % of normal within 25–30 days. A further

reduction to about 80% of normal is reached after 50–100 days and maintained until nearly 150 days after injury. By 200 days most nerves recover normal velocities, and there is no further change in the subsequent 240 days. Only two operated nerves showing a higher velocity than the control nerves have been encountered, one being 109% of normal 273 days after a crush, and the other 108% of normal 347 days after suture. The changes in these last two animals are perhaps rather larger than should be

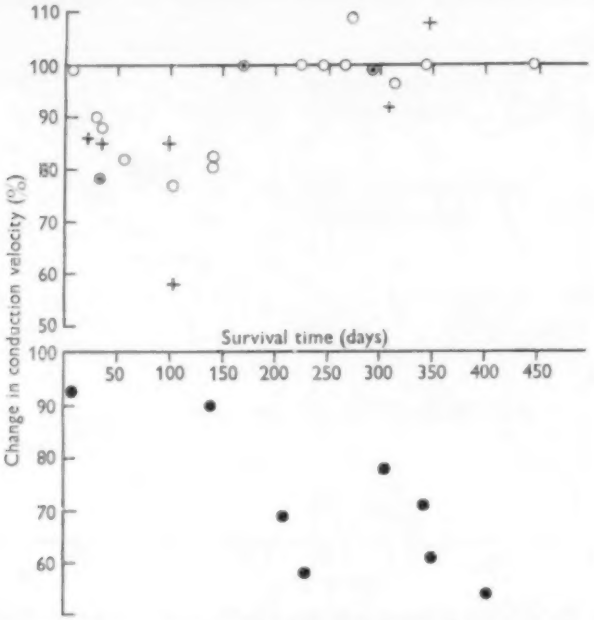

Fig. 3. The conduction velocity of the injured nerve expressed as a percentage of that of the control nerve plotted against survival times after the following injuries: crush ○, constriction ⊙, section and suture +, neuroma ●. The neuromas are plotted separately and, unlike the other injuries, show no recovery.

expected to occur through natural variation and errors of measurement, but are not correlated with especially large axon or fibre diameter (see below), so that their significance is uncertain.

It will be seen from Fig. 3 that the nerves that formed neuromas showed a greater reduction of conduction velocity than nerves subjected to other injuries. The velocities were only 60–70% of normal 200–400 days after the injury, and the trend does not suggest that recovery would ever occur.

Changes in fibre diameter

The changes in total fibre diameter (D) and axon diameter (d) for the different survival times are shown in Fig. 4. Each point is the mean of 60 measurements. As an index of the alterations in the thickness of the myelin sheath relative to axon diameter, the changes in the ratio D/d have also been plotted. This is the reciprocal of the ratio g introduced by Schmitt & Bear (1937).

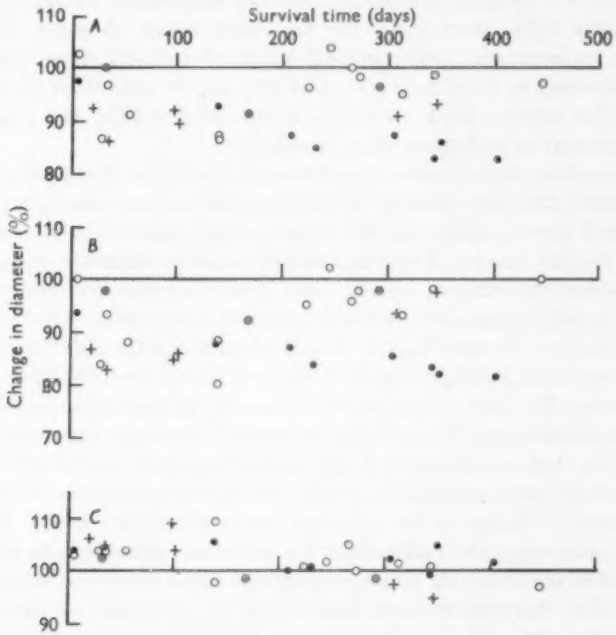

Fig. 4. *A* The total fibre diameter (D), *B* the axon diameter (d), and *C* the ratio (D/d) of the injured nerves expressed as percentages of the corresponding dimensions of the control nerves plotted against survival times. Symbols as in Fig. 3.

For survival periods of 7–150 days the changes are similar for all types of lesion, although the observations were predominantly obtained from crush and from section and suture experiments. During the period 7–50 days axon diameter declines sharply (Fig. 4*B*), total fibre diameter becoming diminished to a lesser extent (Fig. 4*A*). The mean reduction in axon diameter over this period, as compared with the control nerves, is 8·9 % and that for total fibre diameter is 5·3 %. The myelin sheath becomes thicker, showing a mean absolute increase, as compared with the control

nerves, of 5·9%. Thus, with respect to the changes in total fibre diameter, the increase in myelin-sheath thickness partially compensates for the reduction in axon diameter. The increase in myelin thickness relative to axon diameter is evident in Fig. 4*C*. Between 50 and 150 days there is a mean reduction in axon diameter of 14·1% and in total fibre diameter of 10·2%, compared to the control side. The mean value for the absolute thickness of the myelin sheath is 3·5% greater in the operated nerves over this period.

For survival periods exceeding 150 days the results for the neuroma experiments differ from those for the other types of lesion. For the neuroma experiments, with survival times of 208–403 days, there is a mean reduction in axon diameter of 16·1% and in total fibre diameter of 15·0%. The relative thickness of the myelin sheath (Fig. 4*C*) is approximately normal or perhaps a little increased.

For the other lesions axon diameter and total fibre diameter increase, so that after 225 days there is little difference between the operated and unoperated nerves, although the fibres on the operated side remain, in general, slightly smaller. There is little difference between the crush, suture and ligature experiments except that fibre diameter for the two late survival suture experiments (308 and 346 days) is somewhat lower than that for the other two types of lesion. It is of interest that the constricted nerves showed the same reduction in diameter and subsequent recovery as after crush lesions. Evidently the increase in diameter of fibres immediately above the lesion observed by Weiss & Hiscoe (1948) does not prevent the reduction in diameter that occurs higher in the nerve during this period after injury.

The results were examined to determine whether there was any gradient of anatomical change in the operated nerve above the lesion. This was done by comparing the values from the proximal, intermediate and distal levels above the lesion for all experiments in which there was a diminution in total fibre diameter or axon diameter of 10% or more on the operated side as compared with the control nerve. There was no systematic change along the nerve between the three levels, and when the values were compared statistically none of the differences between levels was found to be significant at the 5% level of probability.

The relationship between conduction velocity and fibre size

Providing that it is legitimate to compound the results obtained from all four types of lesion, certain conclusions as to the relationships between conduction velocity and the histological dimensions can be derived. A clear relationship between conduction velocity and axon diameter emerges. Thus, during the period between 7 and 150 days after injury both conduction velocity and axon diameter become reduced, whereas myelin

thickness is increased. Subsequently, except in the neuroma experiments, velocity, axon diameter and myelin thickness return to normal. In the later neuroma experiments velocity is further reduced and is associated with a reduction both of axon diameter and myelin thickness.

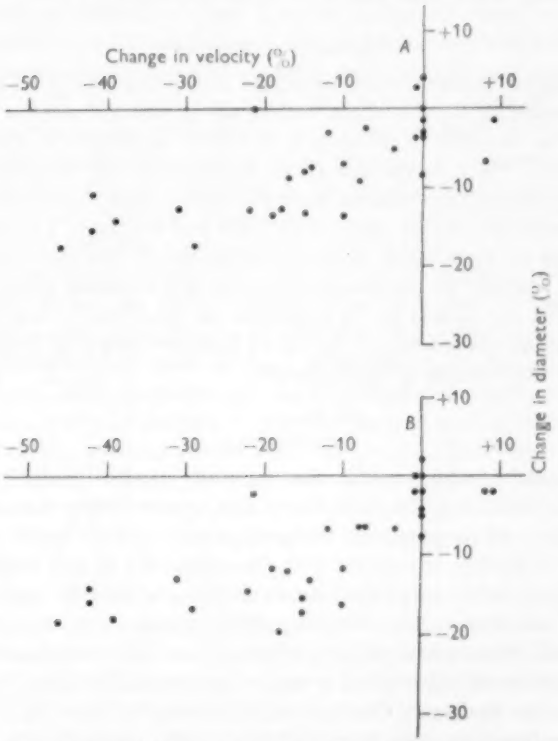

Fig. 5. The percentage difference in conduction velocity between the injured nerve and the control nerve plotted against the percentage difference (*A*) in total fibre diameter and (*B*) in axon diameter.

There is an indication that conduction velocity may also show some correlation with myelin thickness. When the results for the neuroma experiments with long survival times (200–400 days) are compared with those for all types of lesion with shorter survival periods (50–150 days), it will be seen (Fig. 4) that axon diameter is only slightly less, whereas total fibre diameter is reduced to a greater extent because of the difference in myelin-sheath thickness. Yet conduction velocity is substantially less in the former (Fig. 3). The suggestion that conduction velocity may be related

to the thickness of the myelin sheath as well as to axon diameter is supported by Fig. 5, where the percentage change in conduction velocity has been plotted against the percentage change in total fibre diameter (Fig. 5*A*) and axon diameter (Fig. 5*B*). The relationship with total fibre diameter is more nearly linear than that with axon diameter.

The nodes of Ranvier

The nodes of Ranvier were examined in longitudinal sections stained by the Weigert method in eight nerves, all of which showed a reduction in total fibre diameter of between 8 and 18% as compared with the unoperated side. They comprised crush experiments 29, 56 and 102 days after injury, suture experiments 35 and 102 days after injury and neuroma experiments with survival times of 231, 350 and 403 days. The nerves were examined at all three levels proximal to the lesion. No abnormal appearances were noticed. In particular, the nodal gap between adjacent myelin segments did not appear to be increased, as observed by Sanders (1948) in regenerating nerves below the lesion and by Causey & Palmer (1952) during the early stages of degeneration.

DISCUSSION

The present results confirm the previous reports, notably those of Gutmann & Holubář (1951) and Kiraly & Krnjević (1959), that conduction velocity becomes progressively reduced in the central stump after peripheral nerve section if contact with the periphery is not re-established. Gutmann & Holubář noted that the reduction was at first rapid and later more gradual. For lesions following which contact with the periphery is achieved, the situation is clearly different. An initial diminution in conduction velocity is followed by a return to normal. We have been unable to confirm the finding of Sanders & Whitteridge (1946) that velocity is slightly increased between 56 and 123 days after such a lesion.

Numerous observers have noted that fibre diameter in the central stump becomes reduced during regeneration (Greenman, 1913; Gutmann & Sanders, 1943; Weiss, Edds & Cavanaugh, 1945; Sanders & Young, 1946; Sanders, 1948; Gutmann & Holubář, 1951; Eccles & McIntyre, 1953). These investigators confined their examination to distances not greater than 2 cm above the lesion, or did not specify the level of examination. The present observations demonstrate that the changes extend for at least 7–8 cm above the lesion, while in man (see introduction) they may extend for 40–50 cm, since conduction velocity is reduced even as high as this above a lesion. It is evident that widespread changes are produced along a nerve fibre by an injury to its peripheral part.

The alterations in axon diameter and the thickness of the myelin sheath above crush lesions have been studied by Sanders (1948), who also examined the peroneal nerve of the rabbit. Sanders found that there was at first no change in fibre diameter although axon diameter became diminished, the reduction being compensated by a thickening of the myelin sheath. Between 60 and 100 days after operation axon diameter continued to decline and this was also associated with a reduction in total fibre diameter. After 100 days axon diameter increased, myelin sheath thickness remaining unchanged until 200 days and then diminishing. Even at 300 days, however, by which time total fibre diameter was restored, the sheaths were still somewhat thicker than normal. The changes above crush and above section and suture lesions observed in the present study closely parallel those observed by Sanders, except that total fibre diameter tended to decline rather earlier than was suggested by Sanders.

We have made an insufficient number of observations on neuroma experiments during the earlier period after operation (7–150 days) to decide whether the changes in axon diameter and myelin thickness differ from those found after crush lesions. In the later neuroma experiments (200–400 days), axon diameter and myelin sheath thickness appear to be reduced to an approximately equal extent. This is in conflict with Gutmann & Holubář (1951), who reported a relatively greater reduction in the thickness of the myelin sheath than in axon diameter after section of the peroneal nerve of the rabbit with survival times of this duration. This question is being further investigated (J. T. Aitken and P. K. Thomas, unpublished).

The present observations have shown that in this series of nerves conduction velocity is clearly correlated with axon diameter, and have suggested that it may also be influenced by myelin thickness. The question of the correlation between conduction velocity and the anatomical dimensions of myelinated nerve fibres has been discussed in a previous paper (Cragg & Thomas, 1957). The theory of saltatory conduction leads to the expectation that conduction velocity will be equal to the internodal length divided by the transmission time, that is, the delay between the times at which adjacent nodes reach action potential threshold (see Tasaki, 1953). However, it was shown that it is not possible to express conduction velocity directly in terms of the anatomical variables. Empirically, conduction velocity has been found to be most closely correlated with fibre diameter (see Rushton, 1951; Cragg & Thomas, 1957), although Sanders & Whitteridge (1946) reported that in regenerating fibres velocity was more closely correlated with myelin thickness.

In the present experiments, although internodal length was not examined it is presumed to be unaffected; changes in internodal length are confined

to a distance of a few millimetres above a localized lesion (Weiss & Hiscoe, 1948; Lubinska, 1959). As far as can be ascertained from light-microscopy examination, the nodes of Ranvier show no alteration. However, differential changes in axon diameter and the thickness of the myelin sheath occur. Since the latency of activation of a nerve fibre increases as the strength of an applied electrical stimulus is decreased, a reduction in axon diameter is likely to reduce conduction velocity by increasing the potential gradient from an active node to the following inactive node, as a result of an increase in the distributed resistance of the axon. On the other hand, an increase in myelin sheath thickness may produce an effect in the opposite direction by diminishing current leak across the sheath.

The situation may well be more complex than this in that the changes in axon diameter and myelin-sheath thickness may be accompanied by changes in their electrical properties. Thus Kiraly & Krnjević (1959) have suggested that the absolute increase in the thickness of the myelin sheath above the lesion during the early stages of regeneration described by Sanders (1948) may be due to the uptake of water rather than simply to the formation of additional myelin. This would be likely to reduce its capacitance.

SUMMARY

1. Conduction velocity, axon diameter and total fibre diameter have been measured in the peroneal nerves of rabbits proximal to lesions produced by crushing, constricting, cutting and suturing or cutting and avulsing the distal end of the nerve, 7–446 days previously.

2. After the first three types of injury conduction velocity falls to 90% of normal within 25–30 days, and is further reduced to 80% of normal within 50–100 days. This reduction is maintained until 150 days, but most nerves recover normal velocities by 200 days and remain normal thereafter.

3. When the distal end of the nerve is avulsed, conduction velocity above the consequent neuroma falls to 60–70% of the normal 200–400 days after injury and shows no sign of recovery.

4. After all four types of injury a reduction of axon diameter in the largest fibres above the injury to an average of 85·9% of normal tends to precede an overlapping reduction of total fibre diameter to an average of 89·8% of normal in the period 50–150 days after injury.

5. For the first three types of injury axon and total fibre diameter increase after 150 days and return to near normal by 225 days after injury.

6. After avulsion of the distal end of the nerve, from 200 to 400 days after injury the largest proximal fibres show a further reduction in diameter, total fibre diameter and axon diameter being reduced approximately equally.

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LACTIC ACID RELEASE BY INTRA-ARTERIAL ADRENALINE INFUSIONS BEFORE AND AFTER DIBENYLINE, AND ITS RELATIONSHIP TO BLOOD-FLOW CHANGES IN THE HUMAN FOREARM

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Duff & Swan (1951) have shown that, in man, the continuous intra-arterial infusion of a relatively large dose of adrenaline causes an initial transient increase, followed by a sustained decrease in muscle blood flow. Following the intra-arterial administration of dibenyline the constrictor response to intra-arterial adrenaline is converted into a marked and sustained dilatation persisting throughout the duration of the infusion (Allwood & Ginsburg, 1959; de la Lande & Whelan, 1959). Dilatation in response to adrenaline has been attributed by Lundholm (1956) to the release of lactic acid from muscle consequent upon the glycolytic action of adrenaline; but, though Barcroft & Cobbold (1956) found an increase in venous blood lactate with intravenous infusions of adrenaline in man, Hildes, Purser & Sherlock (1949) had not been able to show such an increase following intra-arterial adrenaline infusions. An increase in forearm blood flow with intra-arterial adrenaline following adrenergic blocking agents was seen by de la Lande & Whelan (1959), and they suggested that adrenaline may release a substance from muscle, which might be lactic acid, which is responsible for the vasodilatation. The present experiments were carried out to ascertain if intra-arterial adrenaline liberated lactic acid, and, if so, whether the response was affected by a prior infusion of dibenyline, and its relationship to the changes in forearm blood flow.

METHODS

Healthy medical students aged 20–30 years were used as subjects. Room temperature was maintained at $21 \pm 1°$ C. Under local anaesthesia venepuncture was performed near the antecubital fossa, using a vein which appeared to drain deep structures rather than skin. A fine nylon catheter was threaded through the needle, which was inserted towards the hand, and the catheter was passed for about 2 in. (5 cm) into the vein. Heparin-saline was infused at the rate of 1 ml./min to prevent clotting. Blood samples for lactic acid estimation were taken at 3 min intervals, the blood being withdrawn into a sterile lactate-free syringe;

the blood was deproteinized as rapidly as possible with 10% trichloroacetic acid to prevent lactate accumulation from glycolysis. A needle was inserted into the brachial artery of the same forearm through which sodium chloride solution 0·9 g/100 ml. was infused throughout the experiment at 3 ml./min. Adrenaline was added to this saline when required so that it entered the artery at the rate of 2 μg/min. In each experiment blood samples were taken for analysis during a control period, during a 10 min infusion of adrenaline and during a succeeding time to allow blood lactic acid to return to approximately the resting value. Dibenyline was then given at a dose of 500 μg/min for 6 min and the experimental procedure was repeated.

Blood flow was measured by venous occlusion plethysmography (Barcroft & Swan, 1953). Blood lactic acid was determined by Hullin & Noble's (1953) modification of the method of Barker & Summerson (1941).

RESULTS

Adrenaline infusion. With adrenaline administered intra-arterially at 2 μg/min the lactic acid content of the venous blood draining the limb rose gradually; the peak level ranged from 18 to 100% above the resting level with a mean increase of 43%. After the end of the infusion the concentration of lactic acid returned only slowly to the pre-infusion level and was still elevated 10 min afterwards. Table 1 shows the lactic acid values for the seven subjects used. During the initial transient increase in forearm blood flow there was no change in blood lactic acid, during the sustained fall in blood flow the lactic acid in the blood rose and during the period of 'after-dilatation' seen immediately on stopping adrenaline infusion the blood lactic acid was still high (Fig. 1).

TABLE 1. Lactic acid content (mg/100 ml.) of venous effluent from forearm muscles before, during, and after intra-arterial infusions of adrenaline (2 μg/min)

Subject	Before adrenaline	Minutes after beginning of infusion			Minutes after end of infusion	
		3	6	9	3	9
		Adrenaline				
1	9·0	11·5	10·0	11·5	11·8	11·8
2	8·5	8·0	13·5	17·0	13·5	12·5
3	8·5	15·0	11·5	10·0	7·5	10·0
4	6·0	6·0	6·5	8·5	8·0	8·0
5	9·5	9·5	12·5	11·5	15·0	12·5
6	12·0	12·0	12·0	18·5	17·5	—
7	8·5	8·5	9·0	13·5	11·0	9·5
Mean	8·9	10·0	10·7	12·9	12·0	10·7
		Adrenaline after dibenyline				
1	11·0	11·8	14·8	11·5	7·5	7·5
2	10·5	13·5	10·5	9·5	7·5	7·5
3	9·0	16·0	15·5	15·5	13·5	11·5
4	6·0	7·75	15·0	8·5	8·0	8·0
5	6·5	7·5	7·5	9·5	9·5	6·0
6	13·75	16·0	16·0	22·0	15·0	12·0
7	6·0	6·75	11·0	15·5	7·75	8·5
Mean	9·0	11·3	12·9	13·0	9·8	8·7

Adrenaline after dibenyline. Following dibenyline administration adrenaline caused an increase in the venous lactic acid, the rise occurring more rapidly than with adrenaline alone. The peak levels ranged from 23 to 150 % above resting with a mean increase of 44 %. Following the infusion the blood lactic acid fell within 10 min, in some cases to less than control values (Table 1). The forearm blood flow immediately increased when

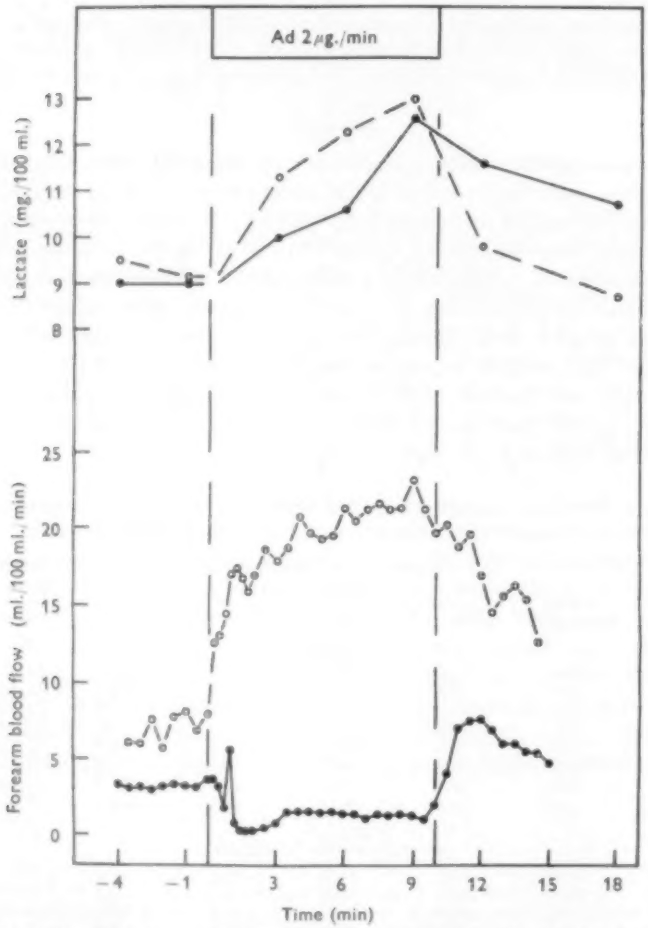

Fig. 1. The effect of I.A. adrenaline 2 μg/min on the venous blood lactate (above) and on forearm blood flow (below). ●, results obtained before treatment with dibenyline; ○, results after I.A. dibenyline (500 μg/min for 6 min). Adrenaline was infused during the period between the broken lines.

adrenaline was administered and before the lactic acid level had changed, it remained elevated and was still above the resting level when the lactic acid had returned to normal (Fig. 1).

DISCUSSION

The intra-arterial administration of adrenaline into experimental animals is known to increase the glycogenolytic activity in muscle, as judged by the increase in the lactic acid content of the blood (Lundholm, 1956; Ellis, 1959). Our results show that this is also true for man when adrenaline is given directly into a muscular segment such as the forearm, and the time course of the rise and fall in lactic acid follows closely that observed for the intravenous infusion of adrenaline in man (Barcroft & Cobbold, 1956). Similar results with intra-arterial adrenaline have been observed by R. F. Whelan (personal communication). These observations do not agree with the findings of Hildes *et al.* (1949), who were unable to detect any change in venous blood lactic acid or any reduction in the glycogen content of muscle from gastrocnemius biopsies when adrenaline was infused directly into the femoral artery.

The increase in lactic acid occurs despite marked degree of vasoconstriction and in this the results in man differ from those in the cat, where Griffith, Lockwood & Loomis (1946) could only find a raised blood lactic acid in response to intra-arterial adrenaline when vasoconstriction was minimal.

The glycogenolytic action of adrenaline in muscle does not appear to be affected by prior treatment with dibenyline. In the present experiments the mean percentage rise in blood lactic acid in response to the intra-arterial administration of adrenaline at 2 μg/min was the same before and after the administration of dibenyline. The time course of the rise and fall of the venous lactic acid was, however, different, in that after dibenyline the lactic acid concentration rose more rapidly and on cessation of the adrenaline infusion the lactic level fell to reach the control level, or slightly below this, within 10 min. The increase in rapidity of these changes may be a reflexion of the greatly increased blood flow through the part when the dilator action of adrenaline is unmasked by dibenyline.

The effect of intra-arterial infusion of adrenaline on the blood flow in the forearm is to cause an initial increase which subsides very rapidly. This initial increase in flow occurs before the lactic acid content of the blood has risen, whether the amine is given intra-arterially as here, or intravenously (Barcroft & Cobbold, 1956), and accordingly it is unlikely that this phase of the adrenaline response is due to the release of lactic acid from skeletal muscle. With intravenous infusions of adrenaline the initial

dilatation is succeeded by a small sustained dilatation and, since this is coincident with the rise in blood lactic acid, it could be related to this in agreement with the postulate of Lundholm (1956). With intra-arterial infusions of small doses of adrenaline (0·1 μg/min) the initial transient dilatation is not followed by a sustained dilatation (Whelan, 1952), and with larger doses (2 μg/min) the secondary response is one of constriction, despite the fact that the blood lactic acid content shows an increase of about 40% above the resting level. Since lactic acid is known to have a dilator action on muscle blood vessels (Green & Kepchar, 1959) this finding implies that any tendency towards lactic dilatation is being opposed by some fairly powerful constrictor activity. This is in accord with the finding of Allwood & Ginsburg (1959) and of de la Lande & Whelan (1959) that in its action on the human forearm adrenaline possesses two components, a sustained dilator and a sustained constrictor action. However, since the blood flow shows some tendency to return towards the resting level during continued intra-arterial adrenaline infusion it is possible that lactic acid may be exerting some small dilator activity.

The sustained dilator action of intra-arterial infusion of adrenaline which is revealed by prior treatment of the forearm with dibenyline is accompanied by muscle glycogenolysis with lactic acid release. This dilatation commences within 1 min of beginning the infusion of adrenaline and is sustained throughout the time of infusion. The blood flow reaches a high level before the lactic acid of the venous blood has risen to any great extent so that it is improbable that the sustained dilatation given by adrenaline following dibenyline is due to the dilator action of lactic acid, although this could contribute towards the slightly increased dilatation later on during the infusion. The lack of correlation between the increase in blood flow and the blood lactic acid level in these circumstances lends support to the suggestion of Allwood & Ginsburg (1961) that it may not be necessary to postulate the formation or release of another dilator substance to explain the sustained dilator activity of adrenaline which is revealed by prior infusion of dibenyline.

This dilatation would represent a continuation of the initial peak dilatation, which is independent of lactic acid release following both intravenous (Barcroft & Cobbold, 1956) and intra-arterial infusion, as our present results show.

When the intra-arterial infusion of adrenaline was stopped before the infusion of dibenyline an 'after-dilatation' occurred (de la Lande & Whelan, 1959; Allwood & Ginsburg, 1961). De la Lande & Whelan (1959) suggested that this could be accounted for by a more rapid wearing off of the constrictor phase of adrenaline action before the dilator effect had ceased. If part of the dilator effect is due to lactic acid release then the

persistence of a raised blood lactic acid level following adrenaline infusion could explain the 'after-dilatation'. This would agree with the finding of Allwood & Ginsburg (1961) that the 'after-dilatation' is specifically related to an action of adrenaline on skeletal muscle, and one certain action is glycogenolysis. Also there was no 'after dilatation' following infusion of noradrenaline, and this does not release lactic acid from muscle. An 'after-dilatation' was absent when the adrenaline infusion was preceded by dibenyline. The 'after-dilatation' might still be due to a dilator effect of lactic acid even if this substance is not implicated in the sustained dilator response to adrenaline. Adrenaline is rapidly destroyed in the circulation, so that its direct action should cease soon after the infusion ceases; the blood vessels would then be exposed to the dilator action of any lactic acid which had accumulated during the adrenaline infusion. Such dilator action would not occur in the absence of lactic acid, as with noradrenaline infusions, nor if the accumulated lactic acid was removed rapidly by a brisk blood flow, as following infusion of adrenaline after dibenyline. At present the 'after-dilatation' is unexplained.

SUMMARY

1. Intra-arterial infusions of adrenaline (2 μg/min) into the human forearm increased the lactic acid content of the blood draining the muscles by an average of 40 %. The same rise occurred when adrenaline was infused after treatment of the forearm with dibenyline (500 μg/min for 6 min).

2. These adrenaline infusions caused an initial transient increase in blood flow which was not related to lactic acid release from muscle. Lactic acid may contribute towards the lessening of the sustained constriction which followed the initial dilatation.

3. After the infusion of dibenyline intra-arterial adrenaline caused only a sustained increase in forearm blood flow which was largely independent of lactic acid release, although a small part of the dilatation could be due to its action.

Our thanks are due to the subjects who took part in the experiments, and to Professor H. Barcroft, F.R.S. for his advice and criticism.

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ANION CONDUCTANCE OF CARDIAC MUSCLE

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The effectiveness of chloride ions in influencing electrical behaviour of a tissue will in general depend on how large a share they contribute to the membrane conductance. In the case of frog skeletal muscle measurements made with different methods are in good agreement and show that the chloride conductance of a resting fibre in Ringer's solution is about twice that of potassium (Hodgkin & Horowicz, 1959; Hutter & Noble, 1960). Experiments on mammalian skeletal muscle (Giebisch, Kraupp, Pillat & Stormann, 1957) and smooth muscle (Burnstock & Straub, 1958) suggest that these tissues are also highly permeable to chloride ions. The present paper gives information on the situation in mammalian cardiac muscle. Although accurate quantitative information has not been obtained, the indication is that cardiac muscle is more permeable to potassium than to chloride. The striking effects which may nevertheless be produced by replacing chloride with other ions are interpreted as the consequence of the sensitivity of certain phases of the cardiac action potential to changes in membrane conductance.

In skeletal muscle the membrane conductance is reduced on replacing chloride by large impermeant anions like sulphate and methyl sulphate, or by bromide, nitrate and iodide which are able to penetrate the membrane slowly (Hutter & Padsha, 1959; Hutter & Noble, 1960). In cardiac muscle the large anions act in the same way, but replacement of chloride by bromide, nitrate and iodide was found to *increase* the membrane conductance. A range of anions of different permeabilities is thus available, and the description of the transient and permanent changes in electrical behaviour which may be produced by varying the anion composition of the bathing fluid occupies part of this paper. A brief report of this work has already appeared (Hutter & Noble, 1959).

METHODS

Strands of Purkinje tissue were taken from the hearts of sheep and dogs as described by Goldenberg & Rothberger (1936) and Draper & Weidmann (1951). The sheep hearts were obtained from anaesthetized ewes which had been used in experiments of a different kind, and were kindly made available to us by Professor Huggett, or from lambs immediately after

slaughter at the cattle market. The preparations were immersed in cold oxygenated Tyrode solution during transport to the laboratory, which usually took about 30 min. Papillary muscles were taken from the right ventricles of anaesthetized cats, or from rabbits immediately after spinal dislocation.

The preparations were mounted in a small channel by means of fine entomological pins inserted through connective tissue into wax. Solutions of the desired composition were forced through the channel at slight pressure from a series of reservoirs equilibrated with 95 % O_2 and 5 % CO_2. The temperature of the solution near the preparation was recorded with an electrically insulated thermocouple and was kept constant at about 35 °C. Control experiments indicated that at most 30 sec was required for 90 % of the bath fluid to be replaced by a new solution. When required, the preparations were stimulated through a pair of fine wire electrodes embedded in the wax.

Membrane potentials were recorded with intracellular micro-electrodes filled with 3 M-KCl. The electrode was connected through a Ag–AgCl half-cell incorporating an Agar–Ringer bridge to one grid of a push-pull cathode-follower input; the other grid was connected to a second half-cell and bridge in contact with the bath fluid. In these circumstances a junction potential of 5 mV (micro-electrode negative) appeared on replacing chloride ions in the bath fluid with methylsulphate ions. For measurement of membrane resistance two micro-electrodes were inserted close together (Fatt & Katz, 1951). In Purkinje strands the insertion of both electrodes usually remained satisfactory for many minutes as judged by the constancy of the membrane potential and resistance in a given solution. When using papillary muscles long-lasting double impalements were obtained more rarely.

The measurements taken were the amplitude of the electrotonic potential close to the polarizing electrode, V, and the total polarizing current, I. For a uniform cable the relation between V and the membrane conductance, g_m, is $V \propto I/\sqrt{g_m}$. Purkinje tissue has a composite structure, but it seems likely that the strands used were long and thin enough for this relation to apply. In papillary muscles, however, the internal current diverges in all directions and a more complicated relation may then be expected to hold. The results are therefore expressed only in terms of the 'effective conductance', I/V.

Chloride solution contained (mM): NaCl 137, KCl 2·7 or 4·0, $CaCl_2$ 1·8, $MgCl_2$ 0·5, NaH_2PO_4 0·36, $NaHCO_3$ 12, glucose 5·5. The other solutions were made by replacing NaCl with equimolar amounts of either NaBr, $NaNO_3$, NaI, $NaCH_3SO_4$ or Na pyroglutamate. The sodium methyl sulphate supplied to us in 1958 by Hopkin and Williams Ltd. was a white crystalline substance almost free from organic odour. In the concentration used it had no obvious 'toxic' effects on frog skeletal and cardiac muscle (Hutter & Noble, 1960) or on the mammalian tissues here used.

RESULTS

The Purkinje tissue of the sheep or dog heart is composed of large, relatively non-contractile fibres. It possesses most of the essential electrical properties of cardiac muscle and is suitable for experiments involving prolonged recording from single cells. In many isolated preparations spontaneous rhythmic action potentials occur, so that the properties of the resting membrane cannot be studied. Some information about the permeability of cardiac muscle to different anions can however be obtained from the changes in rhythm which may be observed.

The action of I, NO_3 and Br on impulse generation

Figure 1 shows the effect of replacement of chloride solution by iodide solution in a spontaneously beating sheep Purkinje fibre. Within a few

seconds of the arrival of iodide solution in the bath the preparation stopped beating and the membrane potential became constant at 10 mV positive to the maximum diastolic potential during activity. The arrest persisted so long as iodide solution was present, but on readmission of chloride solution the preparation soon started to beat again. Artificial stimulation during the arrest elicited propagated action potentials, so that the arrest may be ascribed to an action of iodide on the pace-maker. A possible explanation is that cardiac muscle is more permeable to iodide than to chloride ions. A hyperpolarizing anion current tending to suppress pace-maker activity would then flow on replacement of extracellular chloride by iodide.

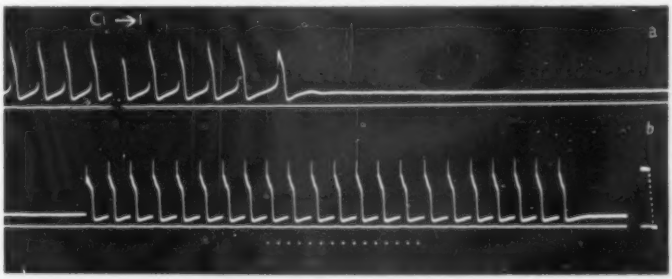

Fig. 1. Suppression of spontaneous activity on replacement of extracellular Cl by I. Sheep Purkinje fibre. *a*, the short break in the record after the fourth action potential indicates the moment at which I solution was admitted. The maximum diastolic potential increases by about 5 mV before the fibre is arrested. *b*, propagated action potentials in response to external stimuli during the arrest. Calibration for this and subsequent similar records, zero potential after withdrawal of micro-electrode from cell and 10 mV steps; time marker, seconds.

When chloride solution was replaced by nitrate solution, spontaneous activity ceased on a few occasions, but usually nitrate failed to cause complete standstill and a more complicated sequence of events then followed. The initial slowing gradually gave way to an acceleration, and on readmission of chloride solution a further increase in frequency occurred before the original rhythm was re-established (Fig. 2). The slowing suggests that nitrate also increases the anion conductance, but to a lesser extent than iodide does. If it is supposed that the anion distribution is determined by the membrane potential, the transience of the slowing and the eventual acceleration produced by nitrate may also be explained (see Discussion).

In a single experiment, short-lasting replacement of chloride solution by bromide solution caused a slowing in the spontaneous rhythm.

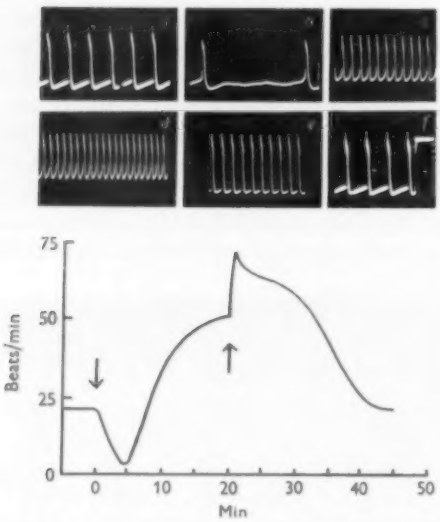

Fig. 2. Time course of frequency changes produced on replacement of Cl by NO_3. Dog Purkinje fibre. *a*, spontaneous activity in Cl solution; *b–f*, sections of continuous record during exposure to NO_3 solution (*b*, *c*) and subsequent readmission of Cl solution (*d–f*). The frequency at different times during the experiment is plotted in the graph. The records illustrated correspond to the following times: *a*, 0 min; *b*, 4 min; *c*, 20 min; *d*, 21 min; *e*, 36 min; *f*, 45 min. At ↓ NO_3 solution admitted, at ↑ return to Cl solution.

The influence of large anions on impulse generation

The interpretation given to the effects of iodide, nitrate and bromide leads to the expectation that anions *less* permeant than chloride should produce the opposite changes. To test this point, chloride solution was replaced by methylsulphate or pyroglutamate solution. Figure 3 illustrates the results of one of these experiments. It can be seen that the first effect of methylsulphate solution was to accelerate the rhythm. Thereafter the fibre slowed progressively until the frequency in methylsulphate solution was 70 % of that in chloride solution. In four other similar experiments the frequency eventually fell to between 40 and 90 %. As might be expected, the initial acceleration produced by impermeant anions was particularly obvious in preparations that had previously been exposed for some time to an anion more permeant than chloride. Replacement of nitrate by methylsulphate, for instance, produced an initial acceleration from 50/min to 120/min and a depolarization in the sense that the maximum diastolic potential reached a less negative value during this period (Fig. 4). Preparations which were

quiescent in chloride solution could often be made to beat by substituting a large anion, and the readmission of chloride arrested such preparations.

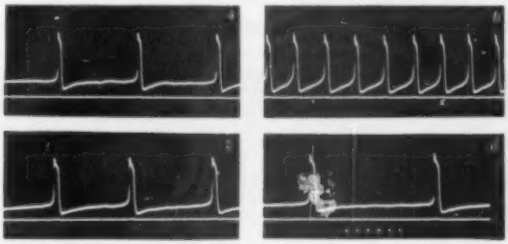

Fig. 3. Transient acceleration and subsequent slowing on prolonged replacement of extracellular Cl by a larger anion. Sections of record from a dog Purkinje cell. *a*, in Cl solution; *b*, 1 min; *c*, 8 min; and *d*, 13 min after admission of CH_3SO_4 solution. Initial displacement of trace in the hyperpolarizing direction due to junction potential change of about 5 mV. Exact values of the maximum diastolic potential at later stages uncertain because of drift in recording system.

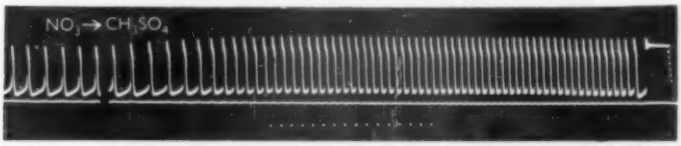

Fig. 4. Dog Purkinje fibre. Break in record after sixth action potential indicates replacement of NO_3 solution by CH_3SO_4 solution. The acceleration is accompanied by a fall in threshold and a depolarization, which is however partly masked by the change in junction potential.

The influence of foreign anions on the action potential

In addition to the rhythm, the plateau phase of the cardiac action potential was found to be sensitive to anion replacement. Comparison of Fig. 3*a* and Fig. 3*c* for instance shows that at similar frequencies the action potential lasts longer in methylsulphate than in chloride solution. A more striking effect of this kind on a sheep Purkinje fibre is illustrated in Fig. 5. In this case replacement of chloride by pyroglutamate solution increased the duration of the action potential from about 0·5 sec to more than 2 sec, with the result that the frequency fell in spite of an increase in the slope of the pace-maker potential. Again much of this effect was transient, though some prolongation of the action potential persisted so long as chloride ions were absent. Occasionally sheep Purkinje fibres were found which had abnormally long-lasting action potentials and the presence of chloride or of another permeant anion was then essential to promote regular repolarization.

Some experiments were made on myocardial fibres in cat and rabbit papillary muscles driven at a constant frequency. Here it was generally not possible to record from a single cell for as long as in Purkinje fibres, but continuous stable records could sometimes be obtained for long enough to

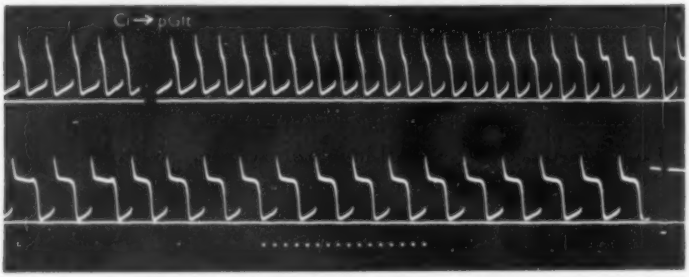

Fig. 5. Prolongation of action potential duration on replacement of Cl by a large anion. Continuous record from a spontaneously beating sheep Purkinje fibre. After fifth action potential extracellular Cl replaced by pyroglutamate (pGlt).

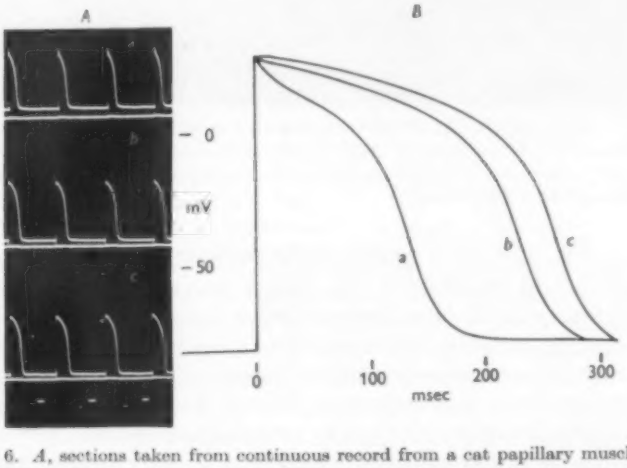

Fig. 6. *A*, sections taken from continuous record from a cat papillary muscle in *a* Cl, *b* NO_3 and *c* CH_3SO_4 solutions. *B*, superimposed tracings of action potentials taken at a faster sweep speed.

indicate that anion replacement has essentially similar effects. In Fig. 6 tracings of action potentials recorded from the same cell in chloride, nitrate and methylsulphate solution are superimposed. Both the initial level and the slope of the plateau may be seen to depend on the anion species present. The terminal phase of repolarization was relatively unaffected.

Changes in membrane resistance

An aim of this work was to determine the contribution of chloride ions to the membrane conductance of cardiac muscle by replacing chloride with impermeant anions. It had initially been expected that all the foreign anions used would decrease the membrane conductance, as in skeletal muscle (Hutter & Padsha, 1959; Hutter & Noble, 1960). The contrasts observed indicated that only methylsulphate and pyroglutamate would be useful for the original purpose, but the opportunity was also taken to examine the changes in conductance underlying the effects of iodide and nitrate.

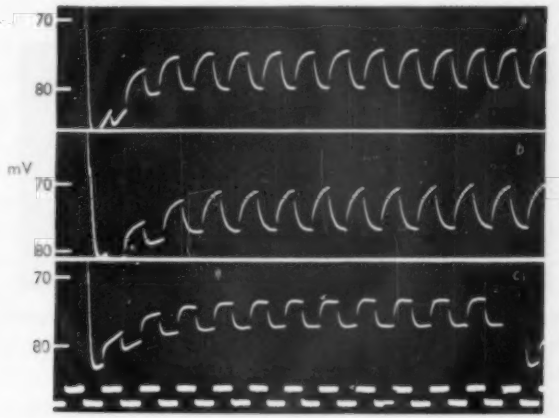

Fig. 7. Variation in resting membrane conductance of sheep Purkinje fibre in different anion solutions. Each trace starts with the down stroke of an action potential and then shows the voltage deflexions produced by recurring hyperpolarizing current pulses of 6×10^{-9} A and 220 msec duration (lowest trace). *a* preparation in Cl, *b* in CH_3SO_4 and *c* in NO_3 solutions. Note that the time course of the downward deflexion at the beginning of each current pulse is faster than the return to the base line, especially in *b*, indicating non-linearity of current-voltage relation.

The normally occurring conductance change during diastole in spontaneously beating Purkinje fibres has been described by Weidmann (1951). In circumstances in which alterations in frequency occur this may be a complicating factor. Figure 7 is chosen from an experiment in which a sheep Purkinje fibre showed periods of quiescence in all the solutions used, so that the conductance of the resting membrane in the presence of different anions could be compared. It can be seen that the voltage displacement produced by recurring hyperpolarizing current pulses of constant intensity is slightly increased on replacing chloride with methyl-

sulphate and clearly reduced by substituting nitrate. The direction of the conductance change is therefore as expected from the alterations in frequency and potential. The quantitative interpretation of the results is complicated by a non-linearity in the voltage–current relation, as indicated by a faster change of potential in the hyperpolarizing than in the depolarizing direction, especially when the voltage deflexion is increased by methylsulphate solution (Fig. 7*b*). A change in sodium conductance with membrane potential probably accounts for most of this effect. On removal of chloride it would tend to increase the change in voltage displacement produced by a given current, so that only an upper limit for the contribution of chloride to the membrane conductance can be obtained. When allowance is made for the cable properties of the fibre, the changes

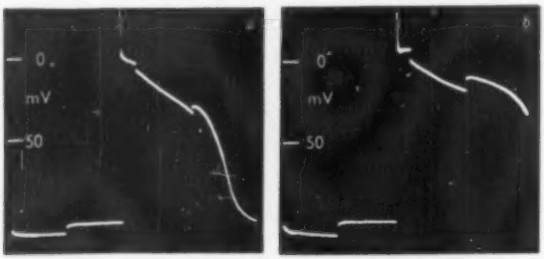

Fig. 8. Dog Purkinje fibre driven at constant frequency. Constant current pulses, 6×10^{-8} A, synchronized to fall in diastole and systole. *a* fibre in NO_3, *b* in CH_3SO_4 solution. Duration of current pulses, 250 msec.

illustrated in Fig. 7 amount to a fall in the membrane conductance, g_m, from 1·0 in chloride solution to 0·7 in methylsulphate solution; g_{Cl} would then appear to be less than 0·3 g_m. Comparison of the membrane conductance in chloride and nitrate solution can also be only approximate and the same applies to giving quantitative expression to the decrease in conductance that can be observed when a fibre arrested by iodide solution resumes spontaneous activity in chloride solution.

On two occasions the slope conductance was measured at the resting potential and during the plateau of the action potential in naturally quiescent Purkinje fibres driven at a constant frequency. The effect of replacement of chloride by methylsulphate was barely perceptible, but a clear difference between the voltage displacements in methylsulphate and nitrate solutions could be observed. In the experiment illustrated in Fig. 8 the contribution of nitrate ions to the slope conductance was approximately the same at the resting potential and during the plateau of the action potential. In the other experiment the anion contribution during the plateau seemed slightly larger than at rest.

The results of chloride removal on resting cat papillary muscle are given in Table 1. On the average the 'effective conductance', I/V, decreased by about 11%. A quantitative estimate of g_{Cl}/g_m cannot be given in this case, but it seems likely that it is of the same order as in Purkinje fibres. Little change in membrane potential was observed at any stage during these experiments, perhaps because of the small size of the fibres and low chloride conductance.

TABLE 1. Values for 'effective conductance' of cat papillary muscles determined alternately in chloride and methylsulphate solutions

Cat	Impalement	Membrane potential (mV)	I/V (μmho)					Mean ratio
			Cl	CH_3SO_4	Cl	CH_3SO_4	Cl	
A	1	80	19·4	17·4	19·6	—	—	1·12
B	2	73	22·2	19·5	22·2	20·0	22·2	1·12
	3	68	11·3	10·5	11·6	—	—	1·09
C	4	65	18·5	17·2	19·3	—	—	1·10
							Average	1·11

The repolarizing action of iodide and nitrate

A proportion of Purkinje fibres kept in chloride solution containing 2·7 mM-K showed a high degree of rhythmic activity associated with a low maximum diastolic potential. In such preparations a marked hyperpolarization accompanied the slowing observed on admission of iodide or nitrate solution (Fig. 9). The character of the record suggests that as the nitrate concentration reaches an adequate value the anion current produces a regenerative change of potential. The phenomenon becomes more striking in fibres so depolarized that rhythmic activity consists in little more than an oscillation in the plateau region (Fig. 10). Under such conditions a small hyperpolarizing current of any origin may produce a striking regenerative repolarization, presumably because of the effects of voltage on the conductances of the membrane to sodium and potassium ions.

DISCUSSION

The anion current during the action potential

It is now recognized that the distribution of chloride ions between skeletal muscle fibres and their environment is determined by the membrane potential, as set by the cations (Hodgkin & Horowicz, 1959; Adrian, 1960). In a fibre that has been resting for some time the chloride equilibrium potential, E_{Cl}, is therefore equal to the resting potential, and only when the potential is displaced does chloride current became a factor in determining electrical behaviour. In a constantly active cardiac-muscle preparation a resting potential does not exist. If the distribution of chloride ions in

cardiac muscle were similarly determined by the membrane potential, E_{Cl} would therefore take up a value intermediate between the maximum positive and negative potentials. The exact value of E_{Cl} will depend on the time course of the action potential and on the voltage–current characteristic

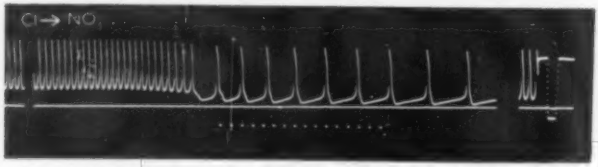

Fig. 9. Hyperpolarization and slowing produced by NO_3 solution. Spontaneously beating dog Purkinje fibre. Change from Cl to NO_3 solution indicated by first break in record. Calibration after return to Cl solution.

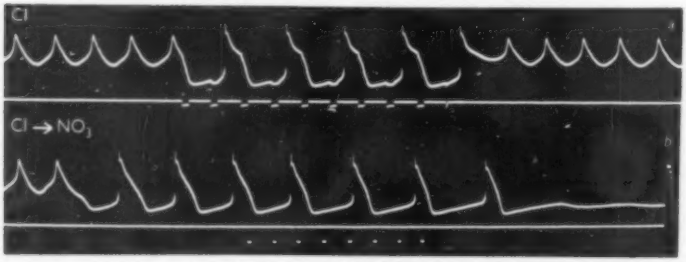

Fig. 10. Restorative effect of hyperpolarizing current pulses and of NO_3 solution on a substantially depolarized sheep Purkinje fibre. *a* fibre in Cl solution shows dwarf action potentials. Brief hyperpolarizing current pulses (8×10^{-8} A) produce regenerative increase in membrane potential and augmentation in amplitude of subsequent action potential. Pulses arriving in diastole have only a small effect. On cessation of current pulses fibre returns to original condition. *b* same fibre immediately afterwards. Increase in membrane potential and transient restoration of fully developed spontaneous action potentials produced by replacing Cl with NO_3.

of the chloride channel, which is as yet unknown in cardiac muscle. In Fig. 11, E_{Cl} has been computed for a Purkinje fibre beating spontaneously at a frequency of 60/min, on the assumption that the movement of chloride obeys the constant field relation as in skeletal muscle (Hodgkin & Horowicz, 1959). The procedure adopted was to determine the inward and outward movements of chloride during the cycle for different assumed values of E_{Cl} until the value at which no net movement occurred was found. In the case illustrated E_{Cl} came to -50 mV. If the chloride conductance did not increase on depolarization, as predicted by the constant field theory, E_{Cl} would have a more negative value. But it seems anyhow likely that E_{Cl} will be found in the threshold region, so that chloride current would contribute to the slow depolarization throughout diastole. During the

plateau, on the other hand, chloride current would flow in the opposite direction and help to terminate the action potential.

That the anion current in fact reverses during the action potential is shown by the changes eventually observed on replacement of chloride by foreign anions. Abolition of the anion current by replacement of chloride by an impermeant anion like methylsulphate slows both the rate of depolarization during diastole and the rate of repolarization during the plateau (Fig. 3*d*). By contrast, when an anion like nitrate, to which cardiac muscle is apparently more permeable, takes the place of chloride in the system a steeper pace-maker potential and shorter action potential eventually occur (Fig. 2*c*).

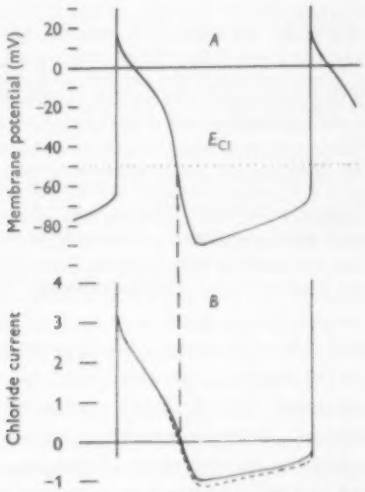

Fig. 11. *A*, voltage time course in a Purkinje fibre beating spontaneously once per second. *B*, time course of Cl current (in arbitrary units) according to equation 2·5 of Hodgkin & Katz (1949) with E_{Cl} taken as -50 mV (solid curve) and -48 mV (interrupted curve). In the first case the time integral of the Cl current over the cycle is zero.

The *initial* changes in rhythm observed on chloride replacement are transient and in the opposite direction to the final effects discussed above. They may be explained as a result of the current that flows so long as anions of different permeabilities are present on the two sides of the membrane. It is convenient to discuss these transient effects with reference to an 'anion potential', E_{An}. This may be defined as the potential at which the net anion current is zero and is given by

$$E_{An} = \frac{RT}{F} \ln \frac{[Cl]_i + \beta[A]_i}{[Cl]_o + \beta[A]_o} \quad \text{(cf. Hodgkin \& Horowicz, 1959),}$$

where R, T and F have their usual meanings, $[\ ]_i$ and $[\ ]_o$ refer to the intra- and extracellular concentrations respectively, A is a foreign anion and β a constant for any given foreign anion which expresses the permeability of the membrane to it relative to the permeability to chloride. In the case where extracellular chloride is replaced by methylsulphate, β may be taken as practically zero and E_{An} becomes positive. A depolarizing current will therefore flow throughout the action potential. The intensity of this current will be proportional to $[Cl]_i$ and will at first exceed the normal depolarizing current during diastole; an initial acceleration may therefore be expected. But as $[Cl]_i$ falls the depolarizing current decreases and the frequency eventually slows to below its original value.

On the basis of the constant-field theory the value of $[Cl]_i$ at which the outflow of chloride into a chloride-free solution equals the net chloride current normally flowing during the pace-maker potential may be calculated. If the effect of electrical activity itself is neglected, it is also possible to make a rough estimate of the rate of loss of chloride into a chloride-free solution, and hence of the expected duration of the acceleration. Taking g_{Cl} as 160 μmho/cm^2, i.e. 20 % of a total membrane conductance of 800 μmho/cm^2 (Coraboeuf & Weidmann, 1954), E_{Cl} as -50 mV and the fibre diameter as 70 μ the expected duration of the acceleration comes to about 2 min. The observed duration was between 3 and 8 min. As activity itself would slow the loss of intracellular chloride, the time course of the acceleration is therefore in reasonable agreement with the hypothesis that it results from the progressive loss of intracellular chloride. In this connexion, it seems likely that changes in frequency through anion replacement comparable to those observed in Purkinje fibres may be difficult to demonstrate in the intact heart because of the small diameter of the fibres of the natural pacemaker.

In the case of nitrate β may be given a value greater than unity and E_{An} will at first become more negative, provided that the change in the anion composition of the external fluid is rapid compared with the exchange across the cell membrane. A hyperpolarizing current will therefore flow during diastole and slow the development of the pace-maker potential.

The usual action of iodide is to produce a permanent arrest. We may suppose that iodide is more permeant than nitrate and that enough hyperpolarizing iodide current flows to suppress diastolic depolarization completely. The absence in this case of an acceleration as the hyperpolarizing current eventually decreases is probably the result of a permanent shift in E_{An} to a more negative value. On the assumption that the anions are passively distributed according to the membrane potential, such a shift would be expected as a direct consequence of the prolonged absence of action potentials. It may be noted that whenever nitrate succeeded in bringing about arrest the eventual increase in frequency was similarly absent.

In preparations not showing pace-maker activity the duration of the plateau was most sensitive to changes in the anion conductance. The indication is that the contribution of anions to the membrane current is

significant only during the slow phases of the action potential when the net cation current is comparatively small. It might be expected that the shortening of the action potential produced by nitrate would be greatest immediately after its admission, while the hyperpolarizing current is most intense. The available evidence, mainly on spontaneously beating preparations, does not illustrate this point perhaps because of the concurrent slowing in frequency which tends to oppose the effect. On replacement of chloride by an impermeant anion the prolongation of the action potential was in fact most pronounced soon after the exchange. This effect was seen especially well in sheep Purkinje fibres, probably because the great diameter of these fibres ensures a slow loss of intracellular chloride.

Comparison with synaptic inhibition

The 'inhibitory' action of iodide and nitrate on spontaneously beating Purkinje fibres resembles in some respects that of weak vagus stimulation on sinus venosus or auricle fibres. In both situations suppression of pacemaker activity goes hand in hand with a decrease in the duration of the action potential, and a substantial hyperpolarization may be observed in preparations showing a low maximum diastolic potential. The two sets of phenomena, however, do not seem to share a common mechanism. In the case of vagus stimulation or action of acetylcholine the hyperpolarizing current is carried by potassium ions and no evidence for an increase in chloride permeability has been found (Harris & Hutter, 1956; Harris, 1959; Hutter, 1961). There are also no serious reasons to think that the effects of iodide and nitrate are brought about indirectly through an increase in potassium permeability. For although such an action may explain the initial slowing or arrest and the shortening of the action potential, it cannot account for the eventual acceleration. Even during the initial phase a significant difference is apparent in the membrane potential at which the preparation comes to rest. In the case of vagal inhibition this potential is always at, or more negative than, the maximum diastolic value (Hutter & Trautwein, 1956). Preparations arrested by iodide, by contrast, show positive after-potentials when stimulated artificially (Fig. 1). This would not be expected if the potassium permeability were appreciably increased. The present results are, however, of some interest in relation to the general problem of synaptic inhibition, for they show how opposite changes may be obtained, depending on the magnitude of the conductance change and the value of the relevant equilibrium potential. They also raise the question whether the central effects of bromide may not be due to a permeability difference, a possibility which is not necessarily ruled out by the lack of central action on the part of iodide because of the known ability of the blood-brain barrier to distinguish between these anions (Davson, 1959).

The anion permeability of natural membranes

The results of replacement of extracellular chloride by methylsulphate on the membrane conductance of Purkinje fibres may be compared with those of similar experiments on skeletal muscle (Hutter & Noble, 1960), and show a quantitative difference between the two tissues. In resting cardiac fibres it may be inferred that chloride ions carry at the most 0·3 of the total membrane current; in skeletal muscle at the resting potential the proportion is as high as 0·68. The origin of this difference is at present unknown, and it may not hold to the same extent at all membrane potentials. When skeletal muscle is hyperpolarized the proportion of current carried by chloride is known to fall to a value similar to that found in resting cardiac muscle, because of an increase in potassium permeability. More information on the voltage dependence of the different ionic currents in Purkinje fibres is required before a complete comparison can be made. It is also not possible at present to give quantitative information on the contribution of chloride to the membrane conductance of myocardial fibres, because the relation between voltage displacement and membrane conductance is uncertain in this case. But the smallness of the changes observed and the similarity with Purkinje fibres in the effect of chloride removal on the action potential suggest that in myocardial fibres also g_{Cl} is relatively low. From the functional point of view, a low chloride conductance makes cardiac muscle more sensitive to changes in cation permeability, whether occurring during the pace-maker and action potentials, or as a result of autonomic nerve activity.

The effects observed on replacement of chloride by penetrating anions illustrate the difference in the permeability of natural membranes to the anions of the lyotropic series. In cardiac muscle the order of permeability is $I > NO_3 > Br > Cl$. The same sequence has been found for the mammalian erythrocyte (Davson, 1959). A reverse order holds for skeletal muscle (Hutter & Padsha, 1959) and probably for frog skin (Mullins, 1958). Protamine collodion membranes resemble cardiac muscle in their selective permeability to anions and this property has been attributed to differences in the degree of adsorption of different anions by the membrane (Sollner, 1949; Sollner & Gregor, 1952). Differences in adsorbability, however, cannot alone explain the situation in cardiac muscle, because they would not account for alterations in membrane conductance when the exchange is between anions whose mobilities in free solution are identical.

SUMMARY

1. Isolated strands of Purkinje tissue and papillary muscles from mammalian hearts have been used to determine the role of Cl ions in the

electrical behaviour of cardiac muscle, and to study the relative membrane permeability to a series of foreign anions.

2. Replacement of extracellular Cl by impermeant anions alters the frequency of spontaneously beating preparations. The time course and direction of these changes suggest that Cl ions normally contribute to the depolarizing current during the pace-maker potential.

3. The duration of the plateau of the action potential is prolonged by Cl removal. It is concluded that the equilibrium potential at which the Cl current reverses lies between the voltage regions occupied by the pace-maker potential and the plateau.

4. Replacement of Cl by I causes arrest of spontaneous activity. NO_3 and Br initially slow the rhythm. These effects are attributed to a hyperpolarizing current resulting from the substitution of extracellular Cl by a more permeant anion. The duration of the action potential is reduced by I, NO_3 and Br.

5. When NO_3 does not cause complete arrest, the initial slowing gives way to an acceleration. It is likely that this transition occurs as intracellular Cl is replaced by a more permeant anion.

6. The membrane conductance decrease observed with two intracellular electrodes in Purkinje fibres when Cl is replaced by CH_3SO_4 indicates that Cl contributes only a small part to the membrane conductance. I and NO_3 increase the membrane conductance.

7. Attention is drawn to the qualitative and quantitative differences between the permeability of cardiac and skeletal muscle to anions.

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RENAL EXCRETION OF HYALURONIDASE AND CALCIUM IN MAN DURING THE ANTIDIURETIC ACTION OF VASOPRESSINS AND SOME ANALOGUES

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Following the earlier observation of Ginetzinsky (1958) on dogs and rats, it was shown (Dicker & Eggleton, 1960) in man that hyaluronidase was present in urine secreted at a slow rate of flow; it disappeared during a water or alcohol diuresis and reappeared when the diuresis was suppressed by injection of vasopressin. Commercial preparations of vasopressin are extracted either from ox or from hog posterior pituitary glands, or from a mixture of both, and are standardized in terms of pressor units. It is known that the chemical structure of hog vasopressin differs from that of ox: the former being lysine-vasopressin, the latter arginine-vasopressin (Turner, Pierce & du Vigneaud, 1951; Popenoe, Lawler & du Vigneaud, 1952). Arginine-vasopressin exists in man, ox, horse, sheep, rat, dog, monkey, camel and marsupials (Acher & Chauvet, 1953; Light & du Vigneaud, 1958; Acher, Chauvet & Lenci, 1959; Sachs & Barret, 1959; Sawyer, Munsick & van Dyke, 1960), whereas lysine-vasopressin exists in hog and hippopotamus only (Popenoe *et al.* 1952; Heller & Lederis, 1960). Physiologically the two vasopressins differ significantly: van Dyke, Engel & Adamson (1956) showed that one pressor unit of lysine vasopressin has but one sixth of the antidiuretic activity of an equal amount of arginine-vasopressin, when assayed by intravenous injection in dogs. A similar discrepancy between the action of the two vasopressins has been observed in the rat (Nielsen 1958; Thorn, 1957, 1959; Sawyer, 1958). It was therefore thought of interest to compare the effects of lysine- and arginine-vasopressins in man. As analogues of vasopressin and oxytocin were made available to us the investigation was extended, and the relative antidiuretic activity of these compounds was determined in man and correlated with the excretion of hyaluronidase. Since Thorn (1960) had recently shown that an increased calcium excretion accompanied the antidiuresis produced by lysine-vasopressin in dogs, this was also determined in man.

METHODS

Fifteen young healthy subjects were used, some on two or more occasions. After a period of at least 3 hr without food or water, diuresis was induced by ingestion of water, following one or two cups of tea, until the water load was of the order of 14 ml./kg body weight; it was maintained at that level throughout the experiment. When the subject was in full diuresis, an intravenous infusion of one or other compound was given, for a period of 10 min usually. When the effect of the first compound was over, and the diuresis was back to its initial level, another substance was infused in similar fashion, thus allowing comparison of the two in the same individual. All subjects were sitting during the experiment, but stood for micturition: urine was collected at 15 min intervals. The temperature of the room varied between 21 and 23° C.

Calcium in the urine was estimated by the method of Bachra, Dauer & Sobel (1958). Repeated measurements of the same solution showed that amounts of 5–50 μg calcium per sample of 0·1–0·2 ml. could be determined with a reproducibility of 2%. Sodium and potassium were estimated by flame photometer. Total osmolarity of the urine samples was measured by the thermo-electric vapour-pressure method of Baldes (1934) modified as suggested by Krogh (1939). Repeated measurements of the same solution indicated that the mean error of a single determination was about $\pm 2\%$, or $\pm 5\%$ if the concentration was less than 250 m-osmole/l. Hyaluronidase was determined by change in viscosity of hyaluronic acid (Dicker & Eggleton, 1960). The molecular weight of the carbowax used in concentrating the urine samples (15,000–20,000) is close to the pore size of the Visking tubing in which it was contained, and with some batches slight leakage occurred through the tubing. The viscosity of these smaller molecules was, however, unaffected by boiling, unlike the original mixture, so that the boiled sample of urine still served as a satisfactory blank in relation to any change in viscosity induced by hyaluronidase in the unboiled sample. In view of the recent criticism of Berlyne (1961) that hyaluronidase may become adsorbed on dialysis tubing, it should be said that with the Visking tubing used in the present work standard hyaluronidase was recovered quantitatively when added to a large volume of water or dilute urine and concentrated in the usual way.

Drugs used for intravenous administration were: arginine^8-vasopressin, arginine^8-oxytocin, lysine^8-vasopressin, lysine^8-oxytocin, phenylalanine^2-lysine^8-vasopressin and oxytocin. The first three were extracts from ox, chicken and pig pituitary glands, respectively. The last three were synthetic products manufactured by Sandoz, Ltd.

Vasopressin analogues as well as lysine^8- and arginine^8-oxytocin were assayed for their pressor activity according to Dekanski's (1952) method against the Third International Standard for posterior pituitary (Bangham & Musset, 1958). They were injected or infused intravenously in doses stated in pressor units. Oxytocin was administered intravenously in doses stated in oxytocic units.

RESULTS

Arginine^8-vasopressin injections or infusions were given on eleven occasions, lysine^8-vasopressin fifteen times, arginine^8-oxytocin six times, lysine^8-oxytocin five times, phenylalanine^2-lysine^8-vasopressin ($= PLV_2$) four times and oxytocin twice, with entirely consistent results. A representative comparison of their antidiuretic activities is shown in Fig. 1. Oxytocin (dose of 19 m-u. of oxytocic activity) shows no activity, a result in agreement with those of other observers (Cross, Dicker, Kitchin, Lloyd & Pickford, 1960). Of the other compounds given in a dose of 19 m-u. of

pressor activity, PLV_2 produces negligible antidiuresis, lysine^8-oxytocin appreciably more, arginine^8-oxytocin and lysine^8-vasopressin considerably more and arginine^8-vasopressin most of all. A more quantitative comparison of the last two substances was made in one individual by giving smaller doses in single intravenous injections. The results given in Fig. 2 suggest that arginine^8-vasopressin has approximately twice the antidiuretic activity of lysine^8-vasopressin.

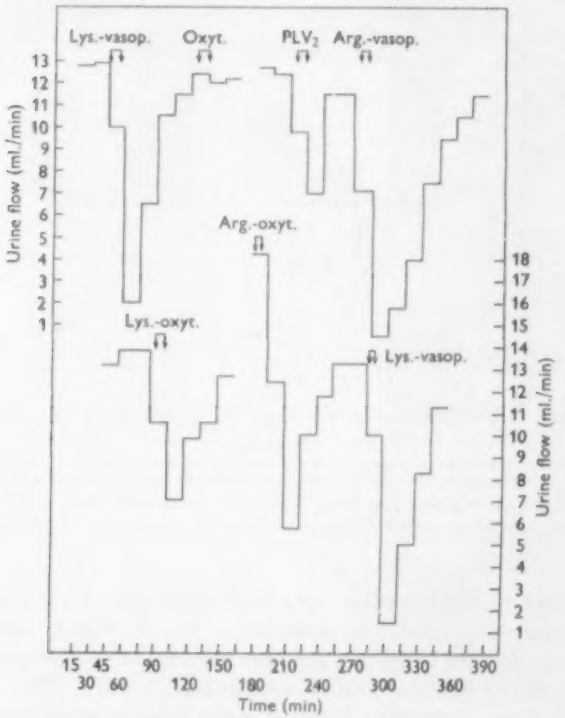

Fig. 1. The relative antidiuretic response to intravenous infusions of 19 oxytocic milliunits of oxytocin and of 19 pressor milliunits of vasopressin and some of its analogues, in four subjects.

Calcium excretion. Calcium excretion varied inversely with the rate of urine flow: it was high at low rates of flow and decreased with the onset of diuresis (Figs. 3 and 4). The higher rate of calcium excretion observed, either at low rates of flow before the onset of diuresis, or during antidiuresis occurred irrespective of the fact that the initial rate of calcium excretion varied widely from one subject to another and on different

occasions in the same subject. The antidiuretic effect following the infusion of vasopressin or one of its analogues was always accompanied by a marked increase of calcium excretion. This agrees with Thorn's (1960) findings in the dog. Moreover, there is a relationship between the magnitude of the antidiuretic effect and the rise of calcium output in the same subject, as may be seen from Fig. 4. Oxytocin which, in the doses used, had no antidiuretic effect did not affect calcium excretion.

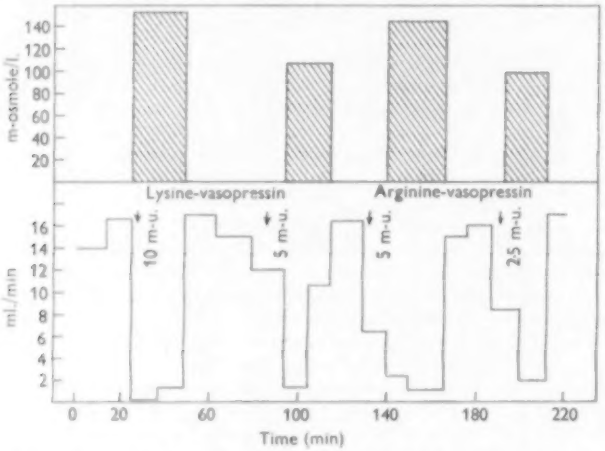

Fig. 2. A comparison of the antidiuretic response to single intravenous injections of varying doses of lysine- and of arginine-vasopressin in one subject. The osmolar concentrations of the urines during antidiuresis are given in the upper part of the figure.

Hyaluronidase concentration. As found previously (Dicker & Eggleton, 1960), hyaluronidase could be detected in the urine at a relatively slow rate of flow, and its concentration was then more or less quantitatively related to rate of flow, as may be seen in Figs. 3 and 5. This relationship was much closer than that between total hyaluronidase excretion and rate of flow, and although individual differences existed they did not obscure an overall relationship, as may be seen from the results shown in Fig. 6.

Na and K excretion. None of the products used increased the excretion of potassium. In regard to sodium excretion, arginine^8-oxytocin alone caused some increase in three out of the six subjects used, in one of whom it was accompanied by an increase in creatinine excretion. Lysine^8-oxytocin produced a decreased excretion of both ions, accompanied by a decreased creatinine output (Fig. 5), suggesting that the antidiuretic

effect observed was partly due to vascular effects. Neither of these two substances had any effect on the blood pressure (measured with a sphygmomanometer) whereas PLV_2 raised both systolic and diastolic pressures.

Total osmolar concentration was measured in a number of experiments and showed the usual inverse relation with rate of urine flow. An example

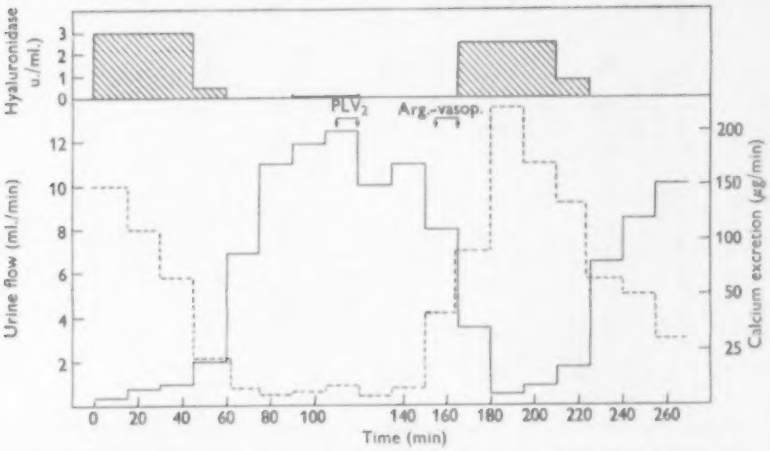

Fig. 3. The relationship between rate of urine flow, Ca excretion and hyaluronidase concentration in the urine before and during water diuresis and after intravenous infusions of 19 pressor m-u. of PLV_2 and of arginine-vasopressin. Ca excretion, – – –.

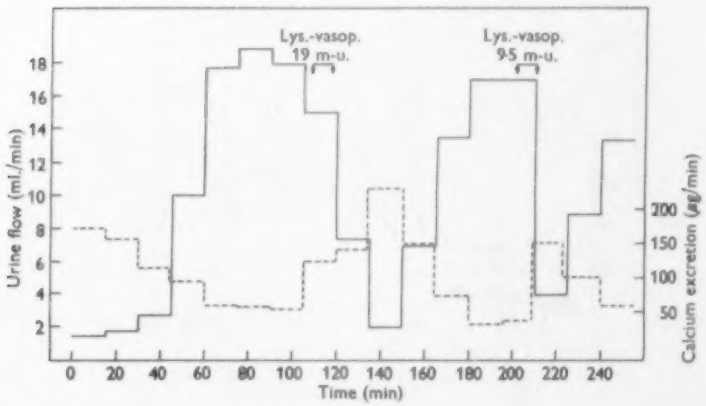

Fig. 4. The relation between calcium excretion and rate of urine flow following intravenous infusions of different doses of lysine-vasopressin. Ca excretion, – – –.

is given in Fig. 2, stressing the similarity of the effects obtained by injections of lysine-vasopressin with those of a half pressor dose of arginine-vasopressin.

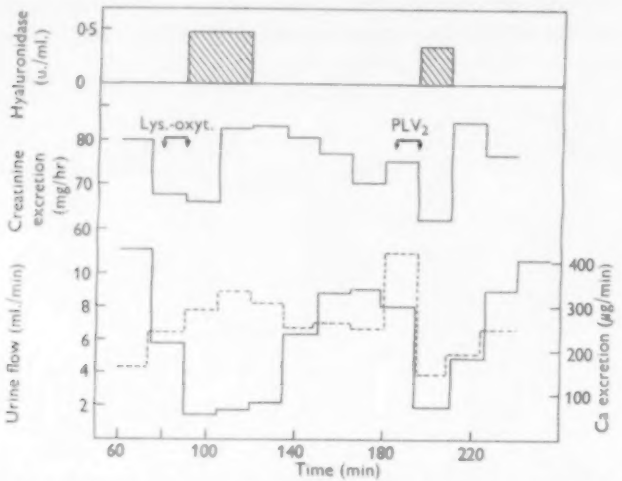

Fig. 5. The effects of large doses (40 pressor m-u.) of lysine^8-oxytocin and of PLV_2 on rate of urine flow, creatinine and calcium excretion, and hyaluronidase concentration in the urine. Ca excretion, – – –.

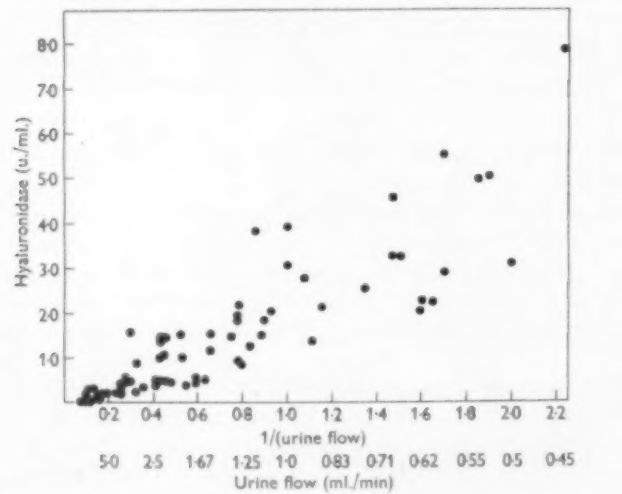

Fig. 6. The relationship between hyaluronidase concentration in the urine and the reciprocal of urine flow in seventeen subjects (seven of the present fifteen and ten others).

TABLE 1. Structural differences in vasopressins and some of their analogues in relation to their antidiuretic activity.

	1	2	3	4	5	6	7	8	9	Antidiuretic activity
Oxytocin	CYS	TYR	ILEU	GLU(NH_2)	ASP(NH_2)	CYS	PRO	LEU	GLY(NH_2)	0
Lysine^8-oxytocin	CYS	TYR	ILEU	GLU(NH_2)	ASP(NH_2)	CYS	PRO	LYS	GLY(NH_2)	+ +
Lysine^8-vasopressin	CYS	TYR	PHE	GLU(NH_2)	ASP(NH_2)	CYS	PRO	LYS	GLY(NH_2)	+ + +
Arginine^8-oxytocin	CYS	TYR	ILEU	GLU(NH_2)	ASP(NH_2)	CYS	PRO	ARG	GLY(NH_2)	+ + +
Arginine^8-vasopressin	CYS	TYR	PHE	GLU(NH_2)	ASP(NH_2)	CYS	PRO	ARG	GLY(NH_2)	+ + + +
Phenylalanine^2-lysine^8-vasopressin (PLV_2)	CYS	PHE	PHE	GLU(NH_2)	ASP(NH_2)	CYS	PRO	LYS	GLY(NH_2)	+

DISCUSSION

The variation of antidiuretic activity in relation to pressor activity of the vasopressins and their analogues examined can be related to the differences in composition of the molecules. In Table 1 they are shown in ascending order of antidiuretic activity. Starting with oxytocin, which in the dose given has no antidiuretic activity, the slight activity of lysine^8-oxytocin is associated with a change of only one amino acid, lysine^8 in place of leucine^8. Considerable further enhancement of activity occurs if either phenylalanine^3 is substituted for isoleucine^3 in this molecule or if leucine^8 of oxytocin is replaced by arginine^8. Introduction of phenylalanine^3 into arginine-oxytocin, giving arginine-vasopressin, approximately doubles its activity. Thus increasing basicity of amino acid, in position 8, would appear to be associated with increasing antidiuretic activity, the effect being enhanced by the presence of phenylalanine^3 in place of isoleucine. This interpretation agrees with the views expressed by Katsoyannis & du Vigneaud (1959). From the observations that the synthetic analogue, leucine^8-vasopressin (= oxypressin) has a low pressor activity (Katsoyannis, 1957), whereas arginine^8-vasopressin has a high pressor activity, Katsoyannis & du Vigneaud (1959) concluded that, 'a strong basic amino-acid in the side chain is one requirement for high pressor activity'. As reported by Acher (1960), the most potent octapeptide in this series of analogues is arginine^8-vasopressin with 600 pressor u./mg, as compared with 300 pressor u./mg for lysine^8-vasopressin, 125 u./mg for arginine^8-oxytocin and 3 u./mg for leucine^8-vasopressin. From the present results it would appear that the antidiuretic activity is even more affected by changes in the molecule, since this has been expressed throughout in relation to pressor activity.

Some indication of the relative importance of amino acids 3 and 8 in the various activities of the analogues may be obtained from comparison of these and other results with our own. Boissonnas, Guttmann, Jaquenoud & Waller (1956) and Berde, Doepfner & Konzett (1957) found that replacement of isoleucine by phenylalanine in oxytocin increased the negligible antidiuretic activity of the latter ninetyfold. A similar replacement in lysine oxytocin also considerably increases its antidiuretic activity. The basicity of amino acid 8, however, seems of even greater importance, for arginine^8-oxytocin has an antidiuretic effect similar to that of lysine^8-vasopressin in terms of pressor units, whereas its pressor activity is only 125 u./mg compared to 300 u./mg for lysine^8-vasopressin.

The analogue phenylalanine^3-lysine^8-vasopressin (= PLV_2 in Table 1) appears to be an anomaly. Its smaller antidiuretic activity may possibly be attributed to the presence of two phenylalanines, in positions 2 and 3,

for tyrosine^2-tyrosine^3-oxytocin has been shown to be an antagonist of oxytocin (Guttmann, Jaquenoud, Boissonnas, Konzett & Berde, 1957).

The definite relationship between hyaluronidase concentration and reciprocal of urine flow shown in Fig. 6 strengthens the suggestion originally made by Ginetzinsky (1958) that it is inherently connected with the antidiuretic action of vasopressin. Berlyne (1960) has criticized Ginetzinky for expressing his results in terms of u./ml. rather than u./min, on the grounds that substances such as creatinine would show a similar relation of decreasing concentration with increasing rate of urine flow when the total output is constant. This is true also in regard to some substances which are largely reabsorbed, as for example sodium and potassium in most of the present experiments. Hyaluronidase, however, is in rather a special category and variability in excretion rate is so great that no convincing relationship with rate of urine flow can be shown. We have already reported such a relationship in any individual experiment, and although there is a day-to-day variation in any one individual, the relationship can be seen in each, as is shown in Fig. 7. At urine flows greater than 1 ml./min there is an unmistakable decrease in hyaluronidase excretion with increasing rate of flow. Fewer determinations were made on the remaining subjects, but a similar relation was found in all of them. No further increase occurs in output per minute at rates of urine flow lower than about 1 ml./min, and this would rather seem to strengthen Ginetzinsky's view that the hyaluronidase appearing in the urine is a spill-over of enzyme not utilized in the walls of the collecting tubules.

Berlyne (1960) finds no such relationship in any of the three subjects studied and his relation between hyaluronidase concentration and rate of flow shows a much wider scatter than that seen in Fig. 6. The most likely cause of this different result is some difference in technique, and although substrate and standard hyaluronidase were of different origin in the two cases dialysis or lack of it would seem to be of greater importance. Ascorbic acid, which reduces the apparent viscosity of hyaluronic acid (McClean & Hale, 1941), is likely to be present in varying amounts in different urines and will vary in amount in the boiled control according to its pH, so that dialysis with subsequent adjustment of electrolyte concentration should provide the more reliable estimate of hyaluronidase content.

Results now presented indicate that the observed increase in urinary calcium is also implicated in the antidiuretic action of vasopressin. Howard, Wilde & Malvin (1960) have shown with the stop-flow technique in dogs that the site of maximum reabsorption of calcium from the tubular fluid is just proximal to that of sodium reabsorption, i.e. proximal to the probable site of action of the antidiuretic hormone. It would seem likely, therefore, that the increased secretion observed under the action of this

hormone is an actual addition of calcium to the tubular fluid. When hyaluronic acid is depolymerized, its contained calcium is replaced by monovalent cations, but whether diffusion of this calcium into the tubular fluid is sufficient to account quantitatively for the observed increase is not known.

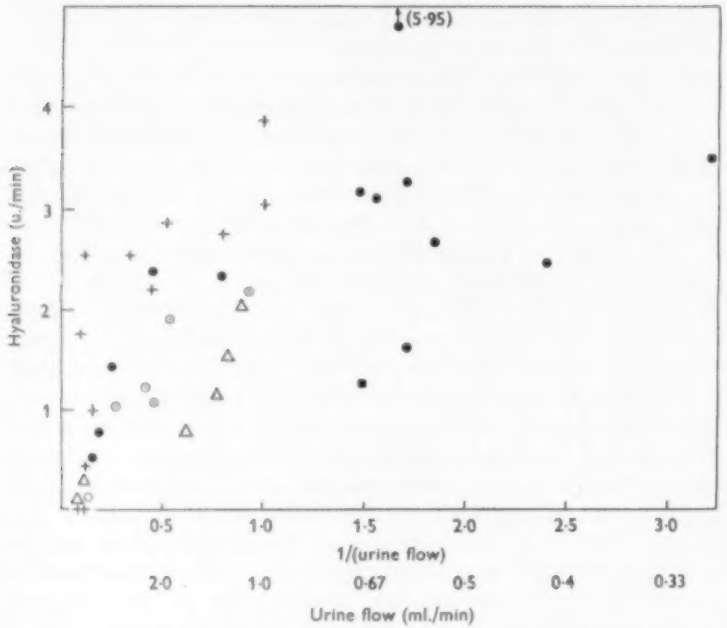

Fig. 7. The relationship between hyaluronidase excretion and the reciprocal of urine flow in four different subjects.

SUMMARY

1. Analogues of oxytocin and vasopressin show different degrees of antidiuretic activity when infused in equipressor amounts intravenously into man during an established water diuresis. Oxytocin has little antidiuretic activity: whereas phenylalanine[2]-lysine[8]-vasopressin, lysine[8]-oxytocin, argine[8]-oxytocin and lysine[8] vasopressin, and argine[8]-vasopressin show increasing activity, in this order.

2. The degree of antidiuretic activity is thus largely determined by the basicity of amino acid[8], with enhancement by phenylalanine[2].

3. The concentration of hyaluronidase found in the urine in these circumstances is quantitatively related to the degree of antidiuresis pro-

duced; antidiuresis is also related to increased excretion of calcium quantitatively in any single experiment.

4. The results support the view that antidiuretic action involves the liberation of hyaluronidase, depolymerizing the intercellular matrix of the tubules in the renal medulla so that the tubular fluid comes into osmotic equilibrium with the hypertonic tissue fluid.

We wish to record our thanks to Messrs Sandoz for the gift of PLV_2, lysine^8-oxytocin and lysine^8-vasopressin and to Professor R. Acher for the gift of arginine^8-oxytocin; to Mrs Margaret Harkness for a supply of hyaluronic acid; to Dr L. E. Bayliss for estimations of total osmolarity and to all the subjects who gave so generously of their time and patience.

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THE EFFECT OF ALTERING THE SKIN TEMPERATURE OF THE LEGS ON THE FOREARM SWEAT RATE

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The study of the afferent system upon which the regulation of sweat production depends is hindered in many circumstances by circulatory adjustments which may alter the thermal topography of the tissues. Thus cooling one region is known to produce reflex vasoconstriction elsewhere, which may alter the temperature of and temperature gradients within the skin of regions other than that deliberately cooled. Since the thermal parameter to which the relevant cutaneous thermoreceptors may be sensitive is unknown, appropriate correction for these effects is impossible. The apparent disagreement between the results of Kuno (1956) and Hill (1921), for example, may be reconcilable on the basis of secondary effects of this sort.

The difficulty may be overcome by working under conditions in which there is no heat exchange from the general body surface. Neither the skin temperature nor the thermal gradients within the skin are then affected by alterations of the cutaneous circulation, and the effects of cooling one region on the rate of sweating from another may be examined on the assumption that the output of receptors elsewhere is unaffected by the procedure.

The present paper reports the results of an investigation conducted under these conditions.

METHODS

The bath. The subject lay in a rectangular metal tank 170 cm by 60 cm by 40 cm deep. This was fitted with an overflow pipe standing 37 cm above the base, which determined the normal working depth of the water. Stirring was provided by a pump situated at one side near the subject's feet. Water taken in at this point was delivered through a closed loop of copper pipe which ran round the bath about 5 cm below the surface of the water, and which was drilled at intervals to distribute the water. Supplies of hot and cold water were led through control valves to pipes terminating near the pump inlet. The temperature of the bath was held equal to the subject's mouth temperature by manual adjustment of the water supplies.

The subject's head was above the water and was enclosed in an insulated box fed with saturated air at the same temperature as the bath water. False walls of aluminium foil were hung inside the box so that the temperature of the surfaces surrounding the subject's head might be assumed to be close to that of the bath. No direct measurement of the rate

of air flow through the box was made, but when ventilation with saturated air at mouth temperature was begun, the skin temperature of the face rose exponentially with a time constant of about 0·7 min.

Temperature measurement. All temperatures were measured by copper-constantan thermocouples wired in groups of five in series, the outputs being fed to a recording potentiometer. The reference junctions were immersed in tubes filled with liquid paraffin and held in a well stirred water-bath controlled to $\pm 0{\cdot}01°$ C. The recorder was sensitive to a potential representing 0·02° C, and results have been expressed throughout to the nearest 0·05° C.

The free couples of the mouth thermometer were individually insulated and passed into small holes drilled in a light alloy cylinder 1·5 cm long by 0·5 cm diameter, which could be comfortably accommodated under the tongue. When this was put in place, the recorded temperature rose exponentially with a time constant of about 0·6 min. The maximum rate of change of mouth temperature in the experiments was about 1·8° C/hr or 0·03° C/min. Errors due to the thermal lag of the thermometer thus do not exceed the other errors of the method. This assessment does not include the thermal lag of the tissues of the mouth except in so far as this is included in the dynamic calibration of the thermometer at a steady mouth temperature.

Sweat rate. Sweat was collected by passing a stream of dry air through a capsule covering 12 cm^2 of the volar surface of the right forearm. The capsule was located on successive days by reference marks tattooed into the skin. A flow of 3 l./min was found sufficient to evaporate all the sweat produced at the highest rates. The effluent air from the capsule was passed through desiccating tubes, which could be weighed at intervals. Ventilation of the capsule was constant throughout and between collection periods, which normally lasted 4 min.

RESULTS

Effect of cooling the legs

The first series of experiments, on subject D.K. only, was designed to compare the forearm sweat rate when the legs were cooled with the reference state in which the legs were not cooled. With the legs immersed and the bath temperature equal to the mouth temperature, the skin temperatures of all regions except the area under the sweat capsule were found to be equal to the bath and mouth temperatures, and all temperatures rose fairly steadily at about 1·8° C/hr. It was found that in this condition the sweat rate from the sample area of forearm was approximately linearly related to the bath, mouth and skin temperature over the range 37–38° C. The legs were cooled by raising them out of the water and supporting the heels on a padded shelf. The degree of cooling could be varied by varying the air movement over the exposed wet skin of the legs and feet. Collection of sweat was begun 1 min after raising the legs out of the bath, and continued for 4 min. Observations in which the legs were cooled were alternated with ones in which the legs were immersed, the timing of the sweat collection being similar.

Results were analysed by plotting sweat rate against temperature (of bath, mouth and skin). A straight line was drawn through the reference points (those in which the legs were not cooled) and it was possible from this line to interpolate the sweat rate which would have been expected in

the other cases had the legs not been cooled. The difference between the sweat rate so interpolated and the observed rate was taken as the depression of sweat rate for the situation concerned. The difference between the bath temperature and the mean skin temperature of the legs during the period of sweat collection was taken as the depression of skin temperature of the legs.

The cooling was associated with a change in posture, which might be expected to produce a change in sweat rate either of fixed magnitude or depending on the existing sweat rate. Thus the depression of sweat rate, ΔS, might be related to the depression of leg temperature, ΔT, according to an equation of the form

$$\Delta S = \Delta T + P + pS,$$

where P is a constant change due to the posture and pS is a change in sweat rate due to posture which is proportional to the sweat rate, S. The effects of posture would appear as significant terms in a double regression of ΔS on ΔT and S, but in practice neither term was significant. A simple regression of ΔS on ΔT gave a regression coefficient of $0{\cdot}44 \pm 0{\cdot}08$ (S.E.) with an insignificant intercept, suggesting that the postural effects were negligible, and the best line through the origin had a slope of 0·48. This relation is shown in Fig. 1.

Effect of arresting the circulation to the cooled legs

The second series of experiments compared the effect of cooling the legs with their circulation arrested and free. The circulation was arrested by inflating large cuffs round the thighs to 200 mm Hg. When the legs were cooled with their circulation free, the skin temperature fell rapidly at first, but soon levelled off and there was little change during the 3rd to 5th minute. The sensation of cooling followed a similar course, being scarcely appreciable by the end of the period. With the circulation arrested, the fall in skin temperature continued throughout the period of cooling, and the sensation remained fairly constant.

The experiment required four types of observation, which were presented in a formal block of 6 experiments on each of 3 subjects. Each experiment consisted of 3 reference observations (legs immersed, thigh cuffs not inflated) between which were interposed observations under two of the following conditions: legs cooled, circulation free; legs cooled, circulation arrested; circulation arrested, legs not cooled. Each pair of these conditions was applied in each order, so that 6 experiments were carried out on each subject. The results for subject D.K. are shown in Fig. 2, from which it will be seen that cooling the legs with their circulation free always depressed the sweat rate, but that cooling the legs with their circulation

arrested did not depress the sweat rate. Arresting the circulation to the uncooled legs was also without effect. Results on the other two subjects were similar.

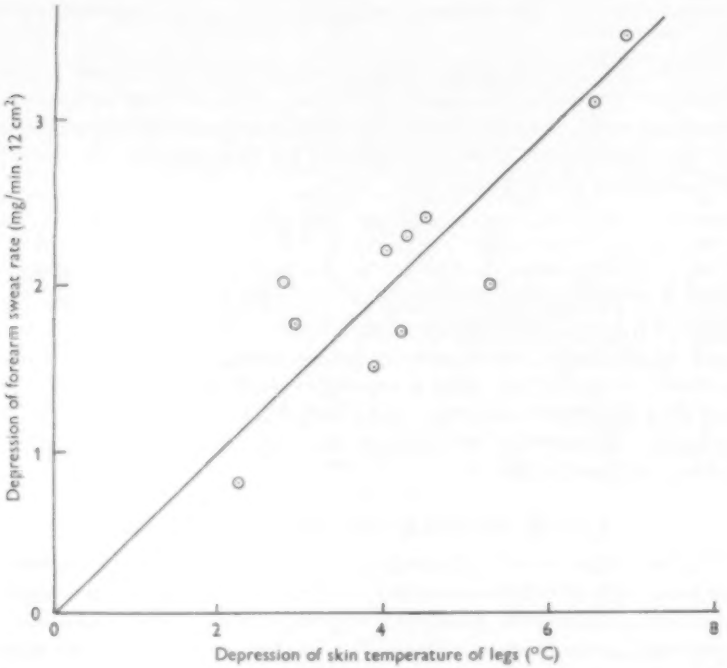

Fig. 1. The effect of cooling the legs on the forearm sweat rate. The method of calculation of the depression of sweat production is described in the text.

The findings are summarized in Table 1, which shows the mean depressions of sweat rate for the three subjects in each situation. The small increases of sweat rate occurring in the situations in which the circulation was arrested are not statistically significant. At temperatures above 38° C the rate of increase of sweat rate with temperature becomes smaller, and the relation is probably curvilinear over the range used for the present experiments. If so, and if there were, in fact, no change in sweat rate in the situations referred to, the method of calculation used would give small negative depressions in these cases.

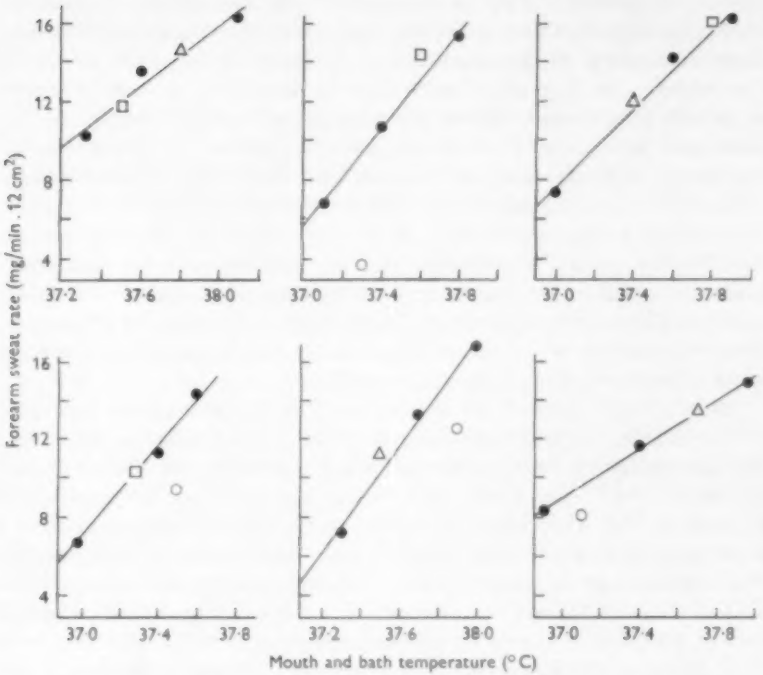

Fig. 2. The effect of cooling the legs on the forearm sweat rate. ○, legs cooled, circulation free; □, legs cooled, circulation arrested; △, circulation arrested, legs not cooled; ●, circulation free, legs not cooled.

TABLE 1. Depression of forearm sweat rate associated with different treatments of the legs. Depression of sweat rate is referred to the situation in which the legs were not cooled, neither was their circulation arrested. Means of 12 observations on 3 subjects

Treatment of legs	Depression of forearm sweat rate (mg/min. 12 cm²)	Significance
Cooled, circulation free	2·74	$P < 0·001$
Cooled, circulation arrested	−0·36	Not sig.
Not cooled, circulation arrested	−0·15	Not sig.

DISCUSSION

The results presented in Table 1 suggest that cooling the legs depresses the forearm sweat rate, and that this effect is abolished when the circulation to the legs is arrested. This could be explained most simply by supposing that the thermoreceptors in the skin of the legs do not contribute to the control of forearm sweat rate, and that the return of cool blood from the

legs to the general circulation accounts for the depression of sweat rate which accompanies cooling of the legs when their circulation is free. Since evaluation of the depression is made with reference to mouth temperature, the depression itself must be referred to the rate of change of mouth temperature. When the circulation is free, cooling the legs reduces the rate of rise of mouth temperature, whereas when the circulation is arrested, cooling is without effect on this. The effect of rate of change of mouth temperature may be ascribed either to a direct action on the deep temperature-sensing mechanism, or to a hypothetical difference between the recorded mouth temperature and the hypothalamic temperature, a difference which might well be expected to depend on the rate of change of either. The results of Benzinger (1959) suggest that the rate of change of deep temperature has no direct action on the sudomotor system, and the latter explanation is therefore to be preferred.

Although the present results support Benzinger's contention that cutaneous thermoceptors are not concerned in the control of sweat rate, this proposition is hard to sustain when the results of Nielsen (1938), Robinson (1949), Lind (1960) and others are considered. These investigations show that at constant work rate in the steady state, sweat rate is linearly related to skin temperature, and deep temperature is constant over a wide range of environments. The deep temperature measured was that of the rectum, and not that of the hypothalamus, but it is hard to believe that hypothalamic temperature changed over the required range while rectal temperature remained unaltered. Moreover, Kerslake (1955) found that the increase of sweat rate with skin temperature was scarcely affected by large changes of deep (mouth) temperature.

The conclusion from the present results that the thermoreceptors of the legs do not contribute to the control of forearm sweat rate rests on the assumption that the output from such receptors would be altered by removing the legs from the water-bath and cooling them with their circulation arrested. This is not necessarily so. Bazett (1951) postulated receptors controlling sweat rate which were stimulated by a temperature difference between arterioles and venules situated at a very similar depth in the skin. Such receptors would have no output in the reference state, since no heat was lost from the skin. With the circulation arrested the two sets of vessels would rapidly approach the same temperature, whatever the heat loss, so that the receptor output would again be zero. Equilibration of temperature might well take place within the first minute of cooling, and the sweat produced during this minute in the present experiments was neither measured nor included in the rate observed during the period of cooling.

Alternatively, it may be supposed that the receptors are sensitive to rate

of heat loss, giving zero output for zero heat loss, and that they or the nerves supplying them cease to give an output after arrest of the circulation for 1 min. Such rapid failure has not been observed in other receptors or nerves, and this explanation seems improbable.

It is concluded that the experiments described in this paper fail to demonstrate the existence of thermoreceptors in the skin of the legs which contribute to the control of the forearm sweat rate, but that the absence of such receptors need not necessarily be inferred.

SUMMARY

1. Subjects were immersed in water at mouth temperature, the head being surrounded by saturated air at this temperature. Sweat rate was measured from a small area of the forearm.

2. Forearm sweat rate varied approximately linearly with body temperature over the range 37–38° C.

3. Cooling the legs was found to depress the forearm sweat rate.

4. When the legs were cooled with their circulation arrested there was no depression of forearm sweat rate.

5. It is concluded that the results fail to demonstrate the existence of thermoreceptors in the skin of the legs which contribute to the control of forearm sweat rate, but that the absence of such receptors need not necessarily be inferred.

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THE RESPONSE OF DE-EFFERENTED MUSCLE SPINDLE ENDINGS IN THE CAT'S SOLEUS TO SLOW EXTENSION OF THE MUSCLE

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The relation between the frequency of discharge of a muscle spindle ending and the extension applied to the muscle has been studied under various conditions (Eldred, Granit & Merton, 1953; Granit, 1958; Granit & Homma, 1959*a*; Whitteridge, 1959; Eldred, Lindsley & Buchwald, 1960). There has not, however, been any comparison of the properties of the primary and of the secondary afferent endings of the muscle spindle in this respect; nor is it known whether Whitteridge's finding for spindles in eye muscles, that intrafusal fibre contraction may increase the sensitivity of an ending to stretch, also holds for either the primary or the secondary endings of spindles in limb muscles. These points have, therefore, been investigated in the present work. The frequency–extension relations were determined under dynamic conditions by recording the discharge of an ending while the muscle was slowly stretched at a constant velocity. The use of this method showed incidentally that for de-efferented spindles the response of primary endings to the phasic component of the stretch is much more marked than that of secondary endings. This confirms the findings of Cooper (1959) for spindles with an intact motor supply.

METHODS

Preparation. The experiments were performed on 23 cats. One was decerebrated and the rest were anaesthetized with pentobarbitone sodium (Nembutal; Abbott Laboratories) given intraperitoneally. The discharge of single afferents from muscle spindle endings lying in the right soleus muscle was recorded from thin dorsal root filaments. Soleus was completely isolated from the spinal cord by cutting the L6, L7, S1 and S2 dorsal and ventral roots. The nerves to most other leg and hip muscles, including the lateral head of gastrocnemius, were cut.

Myography and application of stretch. The right tibia was fixed rigidly by pins and the tension in soleus recorded from the cut Achilles tendon by an isometric myograph which could be moved so as to extend soleus. The movement was at constant velocity, variable from about 0·5 mm/sec to about 9 mm/sec, and was produced by a screw thread driven by a speed-controlled motor. The extension was monitored by means of a microswitch which

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was momentarily closed by a cam once per revolution of the screw thread (0·8 mm extension of the muscle). A rate of stretch in the range 2·3–3 mm/sec (usually 2·7 mm/sec) was used for most of the observations. The extension applied was most often 10–14 mm, but in a quarter of the cases a stretch of 16–19 mm was used. The final extension corresponded approximately to the maximum extension possible *in situ*, though it was fairly often a few millimetres beyond this, particularly when the larger extensions were used. The initial tension was usually only a few grams and, particularly when large extensions were used, the muscle hung slack at the initial length in the absence of ventral root stimulation; in all cases the initial passive tension was less than 100 g. There was no systematic difference between the extensions applied while studying primary and secondary endings respectively.

Fig. 1. Example of the type of record on which most of the measurements were made, showing the response of a primary ending (afferent fibre conduction velocity 95 m/sec) to slow extension (2·9 mm/sec). (*a*) tension in muscle; (*b*) discharge of single muscle spindle afferent; (*c*) output of dekatron scaler also recording afferent discharge, and giving a small pulse for every fifth impulse and a large pulse for every tenth impulse; (*d*) signal lights monitoring extension, upper light turned on at beginning of extension and off when the full extension was applied, lower light flashed once for each 0·8 mm increase in the extension. Initially the tension was less than 10 g and the muscle was slack. Final extension approximately 10 mm. (Records retouched.)

Recording. Two cathode-ray tubes were used. Their sweeps were stopped and their static spots photographed simultaneously on moving paper (usually at 3·5 cm/sec). The four beams available were used to display the following: (1) the amplified action potentials of the single afferent fibre studied; (2) the output of a dekatron scaler, of which the input was fed with the action potentials of the single afferent fibre (Harvey, Kay & Matthews, 1960) and which gave a pulse for every fifth and every tenth action potential; (3) the output of the myograph; and (4) time pulses. Most of the apparatus and experimental technique were the same as in two related preceding investigations and further experimental details were then published (Matthews, 1959; Harvey & Matthews, 1961).

Measurement of records. Figure 1 shows a typical record of the response of a primary ending to slow extension of soleus. The relation between the frequency of discharge of the ending and the extension applied to the muscle was determined during the period of stretching by counting the number of impulses in periods of 0·25 or 0·5 sec, centred on the signal marks corresponding to alternate revolutions of the screw thread (i.e. at extension intervals of 1·6 mm); the periods of measurement did not overlap. The spikes were counted with the help of the scaler; sometimes it was impossible to count individual spikes at all, and the scaler alone was used. The discharge produced by the maintained extension on the completion of the stretching was measured over a period of 0·25 sec, centred 0·5 sec after the completion of the stretching when the frequency of discharge of the endings was only decreasing slowly with time. The frequency of discharge occurring when the extension was nearly complete was measured over a period of 0·25 or 0·1 sec and as late as possible, so that it corresponded approximately to the maximum frequency reached during

stretching. It will be noticed that in the example illustrated in Fig. 1 the discharge of the ending did not immediately accelerate at the apparent beginning of the period of stretching. This was because at the low initial tension used there was initially some slack between the links connecting the muscle to the myograph, and this had to be taken up before the muscle actually began to be extended (see Granit, 1958; Matthews, 1959). Thus no particular reliance should be placed on the first few millimetres of the passive frequency–extension relations.

Identification and classification of endings. Muscle spindle afferents were identified on the basis of their behaviour during a twitch of the muscle elicited by stimulation of the muscle nerve (Matthews, 1933). They were classified as primary or secondary endings on the conduction velocity of their afferent fibres, measured by stimulating the muscle nerve and recording the impulse thereby evoked in the dorsal root filament. The conduction distance was measured at the end of the experiment by dissecting out the sciatic nerve and its attached dorsal roots. For clarity in description of the results it has been arbitrarily assumed that afferents conducting above 72 m/sec supplied primary endings, and those conducting at or below 72 m/sec supplied secondary endings (Hunt, 1954). This classification is unlikely to correspond exactly with any histological classification, but appears to be the best that can be done at the moment, as no consistent functional difference between the two kinds of ending has been established (Matthews, 1933; Hunt, 1954; Harvey & Matthews, 1960, 1961). In addition, graphs were plotted relating various properties of the endings to the conduction velocities of their afferent fibres. This should display any systematic difference between the behaviour of primary and secondary endings even if the dividing line between them (in terms of afferent-fibre conduction velocity) is not sharp, for it is probable that the afferents from primary endings are on average of appreciably greater diameter than those from secondary endings (Ruffini, 1898; Barker, 1948; Hunt, 1954; Eccles & Lundberg, 1959). Altogether 55 endings were studied of which 35 were classified as primary endings and 20 as secondary endings. The distribution of the conduction velocities of their afferent fibres may be seen in Figs. 3 and 6.

Ventral root stimulation. The behaviour of some of the endings was studied during stimulation of ventral root filaments. These were stimulated with 'square' pulses of 0·1 msec duration, isolated from earth. The stimuli were supramaximal for eliciting a contraction and were never less than 4–5 times threshold for the most excitable α fibres, and usually more. They probably excited most or all of the γ motor fibres in the filament, for under the present stimulating conditions the threshold of the γ fibres appeared to be lower, relative to that of the α fibres, than in the muscle nerve.

Most often whole ventral roots were stimulated (L 7 or S 1), but subdivisions of a ventral root were also frequently used. The tension produced in the soleus on tetanic stimulation of the filaments used varied from 2·5 kg to a few grams. Very often an ending might be excited by stimulating both L 7 and S 1 and both these might then be studied; in some cases the response to stimulation of several different subdivisions of one root were studied. The effect of stimulation of a given portion of ventral root was, however, only investigated when the stimulation had an obvious excitatory effect on the ending during an isometric tetanic contraction. Excitatory effects were detected for all but 5 endings (3 primary, 2 secondary) out of 35 for which they were sought.

Post-excitatory facilitation. Occasionally the resting discharge of an ending (almost always a primary ending) increased after a period of γ fibre stimulation (usually accompanied by α fibre stimulation). The after-effect of γ fibre stimulation persisted for several minutes and seemed to be the same as that previously described by Hunt & Kuffler (1951) as post-excitatory facilitation and attributed by them to a 'plastic' change in the spindle. The facilitation could be abolished by stretching the muscle (Hunt & Kuffler, 1951), and the results for passive spindles described in the present paper were obtained in the absence of post-excitatory facilitation.

RESULTS

Passive responses

Response during stretching. The frequency of discharge of all the muscle spindle endings studied increased progressively with the extension while soleus was being slowly extended. Figure 2 shows typical frequency–extension relations found for a primary ending (above) and a secondary ending (below) during the period of stretching. In both cases the frequency of discharge increased approximately linearly with the extension, the slope being 8·2 impulses/sec/mm for the primary ending and 4·3 impulses/sec/mm for the secondary ending. It will be noticed also that the primary ending was discharging steadily at the initial length used (initial tension about 10 g), while the secondary ending did not start to discharge until an extension of about 1 mm had been applied, when the tension was about 50 g. Such a difference was typical, for of 36 primary endings only 3 were not discharging at an initial tension of 10 g or less (these three had threshold under 30 g), while of 21 secondary endings only 4 were excited by a tension below 10 g, and 7 had thresholds of over 100 g (threshold tension determined during extension of 2–3 mm/sec).

The higher threshold to tension of the secondary endings has been previously described by Hunt (1954). The mean value of the tension threshold (93 g) in the present experiments was higher than that found by Hunt (19 g), in spite of the fact that the present dynamic determination of threshold would be expected to give a slightly lower value than Hunt's determination of static threshold for maintained firing. Perhaps Hunt cleaned the soleus muscle from surrounding tissue more thoroughly than we did, in which case a given tension applied to the muscle would extend it further, and so would excite its muscle spindles more strongly.

The frequency–extension relation was determined for 55 endings (35 primary, 20 secondary), and for all but one a reasonably straight line could be fitted by eye to the upper part of the frequency–extension relation. Figure 3 shows each value of slope obtained thereby, plotted against the conduction velocity of the afferent fibre of each ending. There is no marked or abrupt difference between the slopes of endings with afferents of high conduction velocity and those with afferents of low conduction velocity. If, however, an afferent-fibre conduction velocity of 72 m/sec is taken to separate primary and secondary endings, then the mean slope for the primary endings is slightly higher than that for the secondary endings (7·6 and 6·0 impulses/sec/mm respectively). Though this difference is just statistically significant ($0{\cdot}05 > P > 0{\cdot}01$, by the t test), the probably more important finding is that the values of slope for primary and secondary endings overlap so greatly.

The values of slope already given were obtained on stretching soleus at between 2·3 and 3 mm/sec. Three-quarters of the endings were also studied with a higher rate of stretch

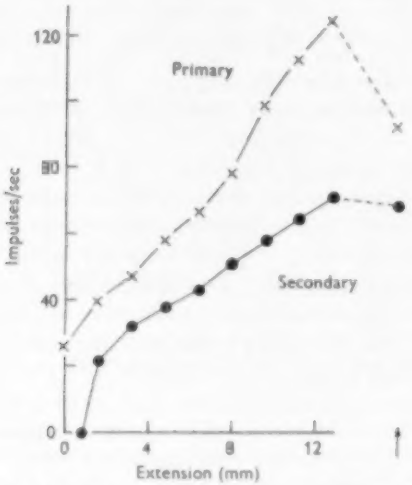

Fig. 2. Examples, for a primary and for a secondary ending, of the relation found between the frequency of discharge and the extension during a slow stretch. The discharge of the primary ending (above, afferent fibre conduction velocity, 88 m/sec) and of the secondary ending (below, afferent fibre conduction velocity 45 m/sec) were recorded simultaneously while applying an extension of 13 mm at 3·0 mm/sec. Abscissa, extension applied at any moment, the arrow marks the discharge found when the full extension had been maintained for 0·5 sec. Ordinate, frequency of discharge of the endings (measured over a period of 0·25 sec).

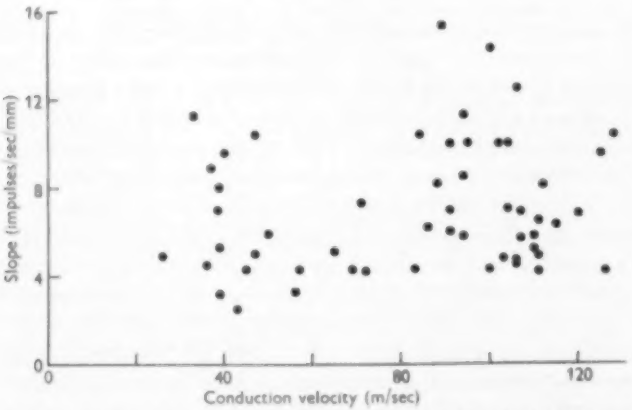

Fig. 3. Scatter diagram relating the slope of the dynamically obtained frequency–extension relations of 54 endings to the conduction velocities of their afferent fibres. (The different results were obtained on stretching at rates between 2·3 and 3·0 mm/sec.)

(usually 7 mm/sec). Increasing the rate of stretch to this limited extent usually produced only a small increase in the frequency of discharge at any extension (mean increase 13%, when the extension was nearly complete). The slope of the frequency–extension relation tended to be slightly greater for the quicker stretch (mean increase 14%), but sometimes the linear upper part of the frequency–extension relation was displaced slightly upwards without any appreciable change in its slope. No clear difference was observed between the behaviour of primary and secondary endings on increasing the rate of stretch in the limited range tested. In a few cases lower rates of stretch (down to 0·5 mm/sec) were used, and then the slope tended to be slightly less than at the standard rate of 2–3 mm/sec. Thus the values of slope already given must have depended upon the choice of 2–3 mm/sec as the standard rate of stretch, but the results obtained would not have been markedly different if a slightly higher or a slightly lower rate of stretch had been used. At higher rates of stretch than were available for the present experiments large effects would be expected on increasing the velocity of stretch, at any rate for the primary endings (Matthews, 1933; Lippold, Redfearn & Vuŝo, 1958; Granit & Homma, 1959*b*; S. Cooper, unpublished).

Slowing of discharge on completion of extension. During the period of stretching the endings were presumably responding to both the static and the dynamic components of the extension. The relative importance of these two factors could be roughly assessed by observing the behaviour of an ending when the dynamic phase of stretching ceased and the final extension was maintained. The frequency of discharge of most endings then fell immediately and 0·1–0·2 sec later reached an approximately constant level which thereafter fell only very slowly, usually by less than 5% in the next 1·5 sec. It is of great interest that the initial slowing was usually much less for secondary endings than it was for primary endings. This difference in behaviour between the two kinds of endings is illustrated in Fig. 4 by records obtained under similar conditions from a single experiment showing the responses of a primary and of a secondary ending on completion of the extension. Figure 5 demonstrates graphically that, though the primary ending fired at a higher rate than the secondary ending during the dynamic phase of the extension, on completion of the extension the position was reversed.

In order to compare the responses obtained for different endings, the slowing on completion of the extension was expressed as a percentage of the increase in discharge, over the resting rate, produced by the stretching when it was nearly complete. This arbitrary measure was used because it indicates the relative importance of the static and dynamic sensitivities of an ending in determining its response to the full extension. If an ending were entirely insensitive to the dynamic component of the stretch the value would be 0%; while if an ending were sensitive only to the dynamic component of the stretch, the value would be 100%. Figure 6 shows the values obtained for the whole series of endings studied; and confirms that endings with small afferent fibres were less sensitive to the dynamic component of the stretching than were endings with large afferent fibres.

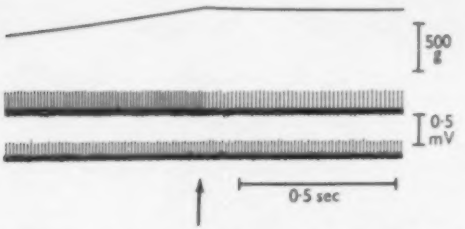

Fig. 4. Records illustrating the greater sensitivity of primary endings, as compared to secondary endings, to the dynamic component of the extension. Top, tension in muscle; middle, response of a primary ending (afferent fibre conduction velocity 112 m/sec); bottom, response of a secondary ending (afferent fibre conduction velocity 39 m/sec). The records show the completion of an extension of 14 mm applied at 2·7 mm/sec. The arrow indicates the point at which the dynamic phase of stretching ceased, and when the final extension was maintained. Both endings lay in the same muscle of the same preparation. Their responses were recorded separately in response to identical extensions, so that only one tension record has been included. (Records retouched.)

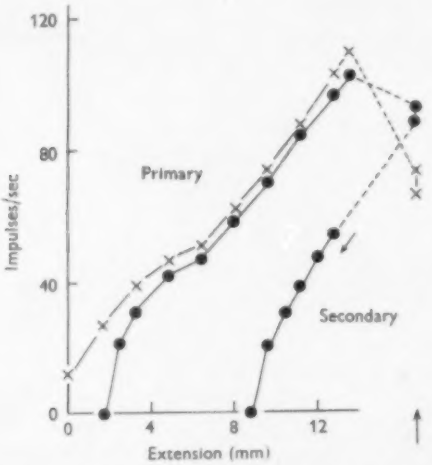

Fig. 5. The full responses to slow stretch of the endings of Fig. 4 shown graphically to confirm their difference in phasic sensitivity. Abscissa, extension applied at any moment, the arrow marks the discharges found when the full extension was maintained (0·5 sec for upper point, 1·5 sec for lower points). Ordinate, frequency of discharge of endings: ×, primary ending; ●, secondary ending. The upper curves were obtained during stretching. The lower curve was obtained for the secondary ending while the muscle was being released; the primary ending did not then fire at all.

If the dividing line between primary and secondary endings is taken as an afferent-fibre conduction velocity of 72 m/sec, then the mean value of the percentage slowing for secondary endings was 11 % (S.E. of mean, 1·9 %, 20 observations) while that for the primary endings was 38 % (S.E. of mean, 2·3 %, 35 observations). The difference between these mean values is highly significant (*t* test, $P < 10^{-6}$).

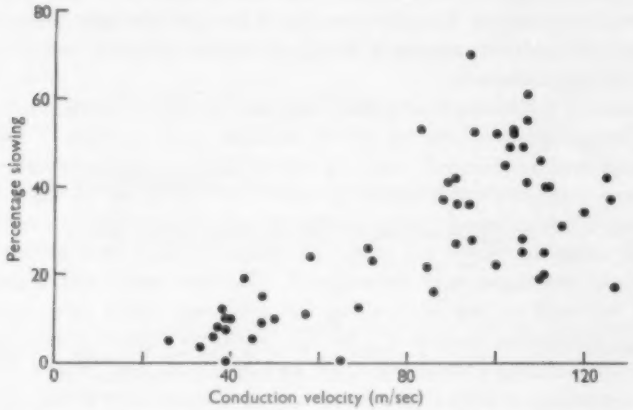

Fig. 6. Scatter diagram relating the phasic responses of 55 endings to the conduction velocities of their afferent fibres. Abscissa: conduction velocity of the afferent fibre of each ending studied. Ordinate: the slowing of the discharge of each ending on completion of the dynamic phase of stretching; this is expressed as a percentage of the increase in discharge, above the initial rate, occurring when the extension was nearly complete. This value gives a rough indication of the importance of the phasic sensitivity of the ending, relative to its static sensitivity, in determining its response to a long slowly applied stretch as this is nearing completion. (Rate of stretch, 2·3–3·0 mm/sec. Total extension, 10–19 mm. See text for discussion.)

The value found for endings with afferents of comparable conduction velocity varies, but no particular significance should be attached to the precise values obtained, for they presumably depended upon both the initial and the final values of the extension employed in each case. There was, however, no systematic difference between the extension applied to primary and to secondary endings. When, for different endings, the slowing of discharge on completion of extensions was compared in terms of its absolute value, in impulses/sec, the differences between primary and secondary endings were equally clear. The observations were made on stretching at 2–3 mm/sec, but the dynamic response of primary endings was still prominent in those cases in which the rate of stretching was decreased to 0·5 mm/sec. Thus, though the percentage slowing, as defined

here, is a rather arbitrary index, the values obtained are thought to show a genuine difference in phasic sensitivity between primary and secondary endings.

A difference in phasic sensitivity of primary and secondary endings was qualitatively apparent also on applying a rapid stretch by pulling the myograph by hand or by tapping the tendon hook. In both cases the consequent increase in the frequency of discharge was more obvious for primary than for secondary endings, but no systematic or quantitative comparisons were made. Results of interest in this respect have recently been obtained by Cooper (unpublished).

Response to maintained extension. Incidental observations were made on the frequency of discharge of 18 endings when a series of different extensions was maintained, and the resulting frequency–extension relations were compared with those obtained for the same endings during stretching. The discharge was measured when it had settled to a relatively constant value about 0·5 sec after the stretching had been stopped and a constant extension was maintained. The extension was sometimes applied by hand by rapidly pulling the myograph back against a fixed stop, and sometimes more slowly by the 'stretcher' which was stopped at a series of different extensions. The two methods gave similar results. For 7 secondary endings the frequency–extension curves obtained for static stretch were similar to those obtained from the same endings during slow stretching. This would be expected, because for secondary endings the fall in frequency of discharge on completion of the extension is usually small (Fig. 5). The static relation was, however, usually slightly below the dynamic relation, and tended to be of slightly lower slope (mean value 91 % of slope of dynamic relation, range 78–143 %). This is a sufficiently small difference for the results of Fig. 3 obtained by the dynamic method to be taken as applying approximately to the static behaviour of secondary endings. For 11 primary endings, however, the relation of the static curve to the dynamic curve was very variable, and illustrated their greater sensitivity to the dynamic component of the stretch. The static frequency–extension relation of these primary endings was often well below the dynamic relation and their slope was on average only 59 % of that of the dynamic relation (range 25–143 %). The results of Fig. 3 for primary endings cannot, therefore, be taken to apply to their static responses. On the limited results available there is, however, no reason to suppose that there is any large and systematic difference in the slopes of the static frequency–extension relations for primary and secondary endings. The mean value for the 7 secondary endings was 5·4 impulses/sec/mm (range 2·5–8·4) and for the 11 primary endings was 6·1 impulses/sec/mm (range 1·4–15·1).

Behaviour during release of stretch. The behaviour of primary and secondary endings during the release of the stretch at constant velocity was strikingly different. Typical responses are shown in Fig. 7, in which it can be seen that the discharge of the primary ending stopped abruptly at the very beginning of the release, while that of the secondary ending continued during several millimetres of release. Figure 8 correlates, for the whole series of observations, the response of each ending during release with the conduction velocity of its afferent fibre. Of 20 presumed secondary endings 19 fired on release, while of 35 presumed primary endings only

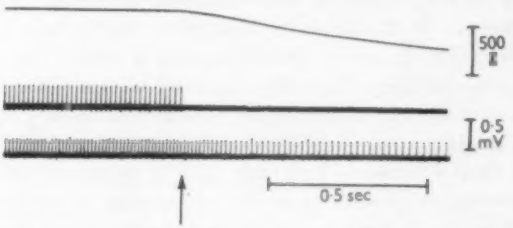

Fig. 7. Records illustrating the contrasting behaviour of primary and secondary endings on release of the extension. Top, tension in muscle; middle, discharge of a primary ending (afferent fibre, 112 m/sec); bottom, discharge of a secondary ending (afferent fibre, 39 m/sec). The records show the first part of the release of the muscle from an extension of 14 mm. The arrow indicates the beginning of the release, which was at 2·7 mm/sec but which did not reach this velocity until after about 0·2 sec. Both endings lay in the same muscle but their responses were recorded separately to identical extensions. Figs. 4 and 5 derived from the same endings. (Records retouched.)

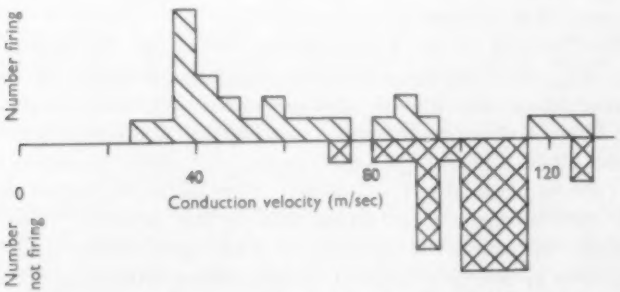

Fig. 8. Histogram relating the behaviour of 55 endings during release of the extension to the conduction velocity of their afferent fibres. Abscissa: conduction velocity of afferent fibre. Ordinate: above, number of endings firing during the release of the extension (most endings fired for several millimetres of release); below, number of endings not firing during release of the extension (none of these endings fired more than three impulses after beginning to release the muscle at 2–3 mm/sec and any such impulse would be in the first 0·1 sec; most fired none).

7 did so, and 3 of these fired a few impulses only. This difference is statistically significant (χ^2 test, $P < 0{\cdot}01$). Secondary endings were not, however, completely unaffected by whether they were being stretched or released, for their frequency of discharge at any extension was always less during release than during stretching (Fig. 5). The magnitude of the difference between the stretch and release frequency–extension relations of different secondary endings varied greatly.

The results of Fig. 8 were obtained on releasing at 2–3 mm/sec, but when higher (7 mm/sec) or lower (0·5–1 mm/sec) velocities of release were tested the behaviour of any ending at the beginning of release was usually unaltered. However, if still faster releases were to be used more endings might be expected to be silent on release, and if still slower releases were to be used more endings might be expected to fire. The behaviour on release was also relatively independent of the final extension applied, which was approximately physiological full extension or a few millimetres more. But if larger final extensions had been used it seems probable that more of the primary endings would have fired on release. In a later experiment (not included in the present series) the responses of two primary endings were studied on progressively increasing the final extension. They began to fire on release when the final extension was 6–8 mm greater than that possible *in situ*, when the passive tension in soleus was 2·5–3·4 kg. The primary endings of Fig. 8, which fired on release, did so for extensions which produced much less tension in soleus (360–1050 g). Yet, however the various endings might have behaved under other experimental conditions, under the present conditions the responses during release of primary and secondary endings differed sufficiently clearly to show a systematic difference in their properties.

Finally, it should be mentioned that the behaviour of different endings on completing the extension and on beginning to release the extension did not appear to be continuously graded with conduction velocity of the afferent fibre. Rather it appeared probable that there were two distinct populations with different behaviour and a fairly sharp transition between them in terms of conduction velocity. For, as is illustrated in Fig. 9, endings with afferent fibres quite close to the arbitrary dividing line (conduction velocity 72 m/sec) might show quite different behaviour, characteristic of the main mass of either primary or secondary endings.

Absence of effect of anaesthetic. 54 out of the 57 endings were studied in cats anaesthetized with pentobarbitone sodium, and it might be suggested that the anaesthetic had significantly altered the response of the endings to slow extension of the muscle (cf. Matthews, 1933). To investigate this possibility the responses of three endings (a primary and two secondary endings) to passive stretch were studied in a decerebrate cat (with the dorsal and ventral roots cut as usual), and the cat was then given a full anaesthetic dose of pentobarbitone (40 mg/kg, intraperitoneally). This did not cause any significant change in the threshold

tensions of the endings, in the form or position of their frequency–extension relations, in the fall in the rate of their discharge on completion of the extension, or in their behaviour on release. There was no doubt that the anaesthetic was absorbed, since the pinna reflex and the decerebrate rigidity had almost completely disappeared 15 min after its injection, while observations on the endings were continued for a further 25 min. Earlier in the same experiment the effect of ether anaesthesia (by inhalation) was studied on the same three endings and a similar absence of effect was found; sufficient ether was given temporarily to paralyse the respiration and to abolish the rigidity. It may also be noted that these three endings were under observation for just over 3 hr without showing any appreciable change in their responses, which suggests that the responses studied reflected relatively constant properties of the endings.

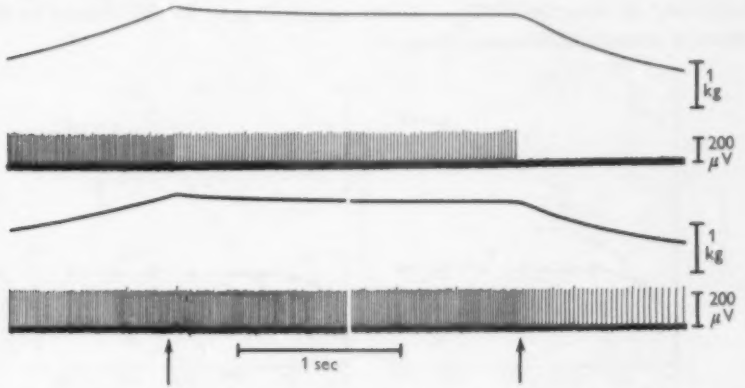

Fig. 9. Records obtained during stretch and release of a primary ending with a relatively small afferent fibre (conduction velocity 83 m/sec), and of a secondary ending with a relatively large afferent fibre (conduction velocity 65 m/sec). The fact that the phasic responses of these endings differ so markedly while the conduction velocities of their afferent fibres do not is in favour of muscle spindle endings being of two functionally distinct kinds, classifiable by the conduction velocity of their afferent fibres, rather than having properties varying continuously with the size of their afferent fibre. (Records obtained from two different preparations. Rate of stretch 2·3 mm/sec in both cases. Records retouched. 1 sec portion removed from record of secondary ending.)

Response during ventral root stimulation

The response of a number of endings to slow stretch was also studied while they were being excited by ventral root stimulation. The ventral root filaments stimulated were large and contained both α and γ motor fibres, so that the response of an ending during stimulation was probably influenced by the contraction of extrafusal muscle fibres (Matthews, 1933), as well as by contraction of its intrafusal muscle fibres. None the less the effect of varying strengths of intrafusal fibre contraction could be investigated in isolation, because the extrafusal muscle fibres of soleus are maximally activated by much lower frequencies of stimulation than are

the intrafusal muscle fibres (Kuffler, Hunt & Quilliam, 1951; Eyzaguirre, 1960). This is illustrated in Fig. 10, which shows typical examples of the effect of different frequencies of stimulation on the muscle contraction and on the discharge of muscle spindle endings. The muscle contraction was nearly maximal at a frequency of stimulation of 30/sec (see also Cooper & Eccles, 1930; Matthews, 1959; Buller, Eccles & Eccles, 1960), while the discharge of both primary and secondary endings increased several-fold when the frequency of stimulation was increased above this value. Thus any differences found in the behaviour of an ending on increasing the frequency of stimulation from 30/sec upwards may be attributed to the effect of intrafusal fibre contraction.

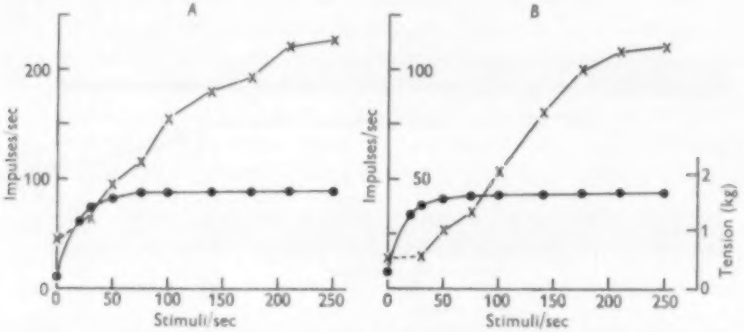

Fig. 10. Examples of the differing effects on muscle contraction (●) and on muscle spindle discharge (×) of increasing the frequency of stimulation. (*A*) for a primary ending (afferent fibre, 95 m/sec); (*B*) for a secondary ending (afferent fibre, 39 m/sec). Abscissa: frequency of supramaximal stimuli applied to peripheral end of L7 ventral root. Ordinates: on left, and centre, frequency of discharge of muscle spindle afferent (measured from 0·5 to 1·0 sec from beginning of stimulation, note different scale for each ending); on right (applicable to both graphs), tension developed in soleus (measured 0·75 sec from beginning of stimulation). Both endings were in the same preparation, but were studied at slightly different initial tensions (50 g for *A*; 160 g for *B*, corresponding to 4 mm greater extension of the muscle. Stimulus 4·5 times threshold for contraction).

The frequency–extension relations obtained by slow stretching during ventral root stimulation were usually still approximately linear, as shown in Figs. 11 and 12 for a primary and a secondary ending respectively. For both these endings increasing the frequency of stimulation from 30 to 100/sec caused an increase in the slope of the frequency–extension relation, showing that increasing the strength of the intrafusal fibre contraction had increased the sensitivity of the ending to stretch (though in these particular cases the actual values are rather higher for the primary ending). In addition, in spite of the extrafusal fibre contraction, there seems little

doubt that the sensitivity of these endings to stretch was greater during stimulation at 100/sec, than it was in the absence of stimulation. Such findings were common, and appreciable increases in slope on increasing the frequency of stimulation above 30/sec were seen in 14 out of 21 primary endings and in 7 out of 9 secondary endings.

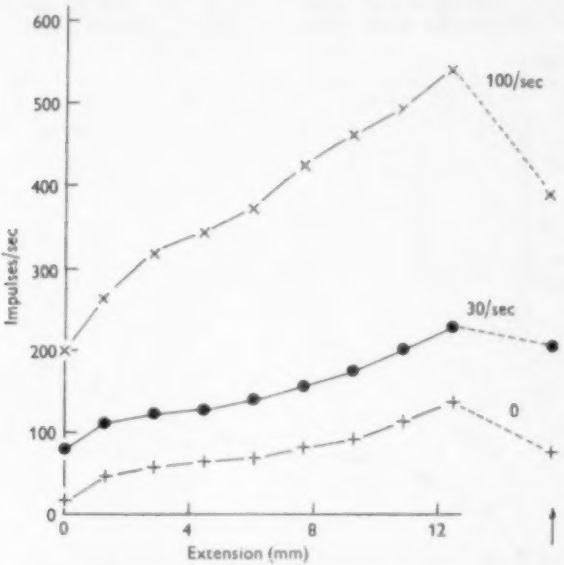

Fig. 11. An example of a primary ending (afferent fibre, 104 m/sec) for which the slope of the frequency–extension relation increased on increasing the frequency of stimulation; +, no stimulation; ●, during supramaximal stimulation of L 7 at 30/sec; ×, during similar stimulation at 100/sec. Slope on 30/sec stimulation 12·5 impulses/sec/mm, on 100/sec stimulation 33 impulses/sec/mm. (Frequency–extension relations determined during stretching at 2·9 mm/sec. The contraction produced 1·6 kg of active tension in soleus. The arrow marks the discharge when the full extension was maintained.)

By saying that the slope increased 'appreciably' it is meant that the slope measured during stimulation at 100 or 160/sec was at least double that found during stimulation at 30/sec; in most cases the slope with 160/sec stimulation was 3–5 times that with 30/sec stimulation. Some endings were studied on stimulation of more than one portion of ventral root and a slope change has then been counted as occurring for the ending in question, even if it was found only on stimulating one of the portions of ventral root. Frequencies of stimulation of 30, 50, 70, 100 and 160/sec were regularly studied.

The maximum slope found for a primary ending during ventral root stimulation was 82 impulses/sec/mm (this was only found over a part of the range of stretching as the discharge of the ending 'saturated' before the end of the stretch), and 11 were found with slopes of 20–45 impulses/sec/mm. The maximum slope found for a secondary ending during

Thus it must be concluded that under the particular conditions studied intrafusal fibre contraction may have rather varied effects on the frequency extension relation, but it does seem important that it frequently increases the sensitivity of both primary and secondary endings to stretch.

Slowing of discharge on cessation of stretching. During ventral root stimulation there was usually still a definite phasic response to the slow stretch, for the frequency of discharge of most endings decreased on completion of the extension (for example, see Figs. 11, 12, 13). For some primary endings, and for many secondary endings, the phasic response seemed to be smaller (both absolutely and as a percentage of the total response to stretch) than in the absence of intrafusal fibre contraction, but for some primary endings the reverse was found; the amount of any such slowing often varied on using a range of stimulating frequencies. The phasic responses of secondary endings were usually obviously less than those of primary endings just as in the absence of stimulation; when a quantitative comparison was made using the same index as before the difference in their behaviour was again found to be statistically significant (calculations made for frequencies of stimulation of 30 and 160/sec).

Stimulation of single γ fibres. The varied results obtained on stimulating ventral root filaments of appreciable size were not due solely to the complications introduced either by the concomitant extrafusal fibre contraction or by the simultaneous stimulation of several different γ fibres influencing the same ending. For in a few cases single γ motor fibres were stimulated in isolation without a clear single pattern of response emerging. The γ fibres were isolated by sub-dividing a ventral root filament, and their conduction velocity was determined by stimulating the muscle nerve and recording their action potential from the ventral root filament. This test also showed that the filament contained only one γ fibre and no α fibres; the absence of α fibres was confirmed by finding that stimulation of the filament produced no detectable tension in the muscle (< 1 g). Four primary endings and one secondary ending were studied on stimulating single γ fibres with conduction velocities ranging from 21 to 41 m/sec. In two cases (one primary ending and one secondary ending) small but definite increases in slope were found on stimulation. For one primary ending no significant change in slope occurred in spite of an appreciable excitatory effect produced by stimulation (two different γ fibres to this ending were stimulated independently with similar results). For the remaining two primary endings there was a change in the frequency–extension relations which could not reliably be described as one either of slope or of intercept. A further three primary endings were studied during stimulation of ventral root filaments containing 1 or 2 α fibres as well as the single γ fibre; for one the slope increased slightly on stimulation, another showed little change in slope, in spite of an excitatory effect of stimulation and the third gave irregular results. The conduction velocities of the γ fibres which caused an increase of slope overlapped with those of the γ fibres which did not. In view of the labour involved it did not, for the time being, seem worth while pursuing these experiments further as they did not appear to be leading to any definite conclusion.

Probable effect of maintained extension. The frequency–extension relation determined by continuous slow stretching is not usually identical with that determined for maintained extensions (p. 278), and it might be suggested that intrafusal fibre contraction would produce a constant type of change in the static frequency–extension relation even though it did not do so for the dynamic relation. This was not investigated, but on indirect evidence

seems improbable, for in those cases in which there was little fall in the frequency of discharge of an ending on completion of the extension it may be assumed that the static frequency–extension relation would have been similar to the dynamic relation. Inspection of the whole series of results obtained by the dynamic method showed that in cases in which the phasic response to slow stretch was small—and this included primary as well as secondary endings—increasing strength of intrafusal fibre contraction sometimes produced changes of slope, and sometimes produced excitatory effects without any appreciable change of slope. It may, therefore, be suggested that both these types of response would also be produced in the static frequency–extension relation as a result of intrafusal fibre contraction. Moreover, effects of each type were found among four endings (three primary, one secondary) for which static frequency–extension relations were obtained with and without stimulation at 100/sec of single γ fibres.

Miscellaneous factors possibly affecting the frequency–extension relation. In particular cases various disturbing factors appeared to be influencing the precise form of the frequency–extension relation. It is thought that they are unlikely to have produced serious distortion in the general patterns of responses which have been described, but as only very few examples have been illustrated it seems important to specify some of the possible complicating factors.

(1) 'Saturation'. In a few cases on stretching during stimulation, particularly for primary endings on stimulation at 160/sec, the frequency of discharge of the ending studied did not increase regularly throughout the period of stretching. Instead, the top part of the frequency–extension relation was almost flat. Various reasons may be suggested for such an effect, but none appears to merit detailed discussion.

(2) Driving. Very occasionally the discharge of the ending tended to follow the frequency of stimulation (see Kuffler *et al.* 1951). Under the present conditions this could be due to the mechanical oscillations of the extrafusal muscle fibres, or to those of the intrafusal muscle fibres, or to ephaptic stimulation of the ending by the muscle action potential. Driving showed itself as a tendency for the frequency–extension relation to run horizontally. The usual smooth increase in the frequency of discharge with increasing extension suggested that driving was not normally significant, but this was not checked by examining on a fast sweep the timing of the spikes in relation to the stimulus. Driving tended to occur most readily when large portions of ventral root were stimulated, and in this case was probably due to ephaptic stimulation.

(3) Fatigue of intrafusal muscle fibres. When the length of the muscle was constant, stimulation of the ventral root produced a discharge of the ending which after the first second or so was fairly steady for 10 sec or more; it is therefore unlikely that fatigue normally occurred during the 4–6 sec required to apply a slow stretch. In a few cases, however, on stimulation at 160/sec the discharge of the ending was not well maintained with time and fatigue may have occurred. On stimulating at frequencies above 160/sec fatigue appeared to be more prominent, and such frequencies of stimulation were not, therefore, regularly studied.

(4) Adaptation. Apart from mechanical factors it is likely that adaptation depends upon a lowered excitability of the ending consequent upon the discharge of a very large number of impulses, for endings may be depressed by antidromic stimulation of their afferent fibres (Matthews, 1933; Paintal, 1959). Thus when the frequency–extension relation is determined by the present dynamic method the top of the curve should be more depressed by adaptation than the bottom of the curve, and if a series of curves obtained during intrafusal fibre contraction are compared the higher curves may be more influenced by adaptation than the lower curves. That such an effect occurred is likely, for after a period of ventral root stimulation the frequency of discharge at the final extension was usually less than before. But adaptation cannot be held responsible for the main finding that the slope of the frequency–extension relation was increased by intrafusal fibre contraction, for adaptation would tend

to produce the opposite effect. The magnitude of any such adaptation effect is, however, uncertain. In the five cases when faster stretches (7 mm/sec) and slower stretches (2–3 mm/sec) were compared during ventral root stimulation, the frequency–extension relation for the fast stretch was slightly above that for the slower stretch, as for the unexcited endings. This may have been partly due to adaptation having a greater effect on the response to the slower stretch, but appears equally likely to have been due to the mechanical properties of the ending.

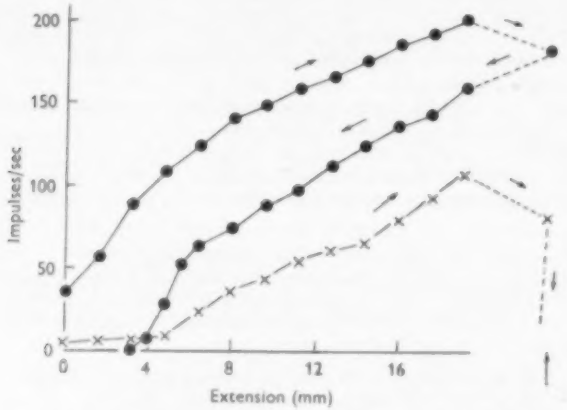

Fig. 14. The contrasting responses of a primary ending (afferent fibre 111 m/sec) during release of stretch in the presence and absence of intrafusal fibre contraction: ×, response of ending in the absence of stimulation; ●, response during supramaximal stimulation of part of L7 at 160/sec. Small arrows indicate whether the muscle was being stretched or released. (Stretch and release both at 2·2 mm/sec. The stretch and release responses during stimulation were obtained in separate stretches, as it seemed undesirable to stimulate continuously for the full period required to determine the relation in a single operation. The maintained frequency of discharge at the full extension was the same in the two cases, and is indicated by the arrow. Contraction tension 150 g.)

Response during release. As already described, in the absence of intrafusal fibre contraction the discharge of primary endings usually ceased abruptly the moment the muscle began to be released from a previously applied extension. When, however, the muscle was released during stimulation of a portion of ventral root or a single γ fibre, the discharge of primary endings continued during part or all of the release. As might be expected, the frequency of discharge at any length of the muscle was lower when the muscle was being released than when it was being stretched. Figure 14 shows typical frequency–extension curves obtained during ventral root stimulation when a primary ending was stretched and then released at the same rate. In the absence of stimulation the discharge of this ending stopped abruptly on release. For this and other endings the firing on

release persisted when the rate of release was increased to about 7 mm/sec. Secondary endings fired on release in the absence of stimulation; during ventral root stimulation they fired on release at a higher rate than in the absence of stimulation, but the discharge on release was slightly below that found at the same extension of the muscle on stimulation during the application of stretch. No quantitative examination was made of the frequency–extension curves obtained by release under different conditions.

DISCUSSION

The most interesting finding of the present work is the difference between the phasic responses of primary and secondary endings. On completion of the dynamic phase of stretching the discharge of primary endings slowed much more than that of secondary endings; and, in addition, on beginning to release the stretch most primary endings stopped firing abruptly, while secondary endings did not. A greater phasic sensitivity of primary than of secondary endings has also been found by Cooper (1959), who worked mainly on spindles with an intact motor supply, and showed further that the difference is also prominent at higher rates of stretch than were available for the present experiments. It would be of interest to confirm all these qualitative findings by measuring some fundamental index of phasic sensitivity, when it might even become possible to classify an ending more accurately by this means than by measuring the conduction velocity of its afferent fibre. The difference in behaviour between primary and secondary endings is probably due to differences in their mechanical coupling to the extension applied to the whole muscle, for the properties of such coupling are thought to influence greatly the phasic sensitivity of mechanoreceptors (Matthews, 1933; Loewenstein, 1956; Hubbard, 1958; Lippold, Nicholls & Redfearn, 1960). The primary ending behaves as if it lies upon a predominantly elastic element which is in series with a considerably more viscous element. The secondary ending behaves as if it lies upon an element in series with another element of similar properties or with only slightly more viscosity. Such suggestions would be quite compatible with the histological finding that the primary ending lies mainly on non-striated or poorly striated regions of the intrafusal muscle fibres, while the secondary ending lies mainly or entirely on striated regions of the intrafusal muscle fibres (Ruffini, 1898; Barker, 1948; Boyd, 1959; Barker & Ip, 1960; Cooper, 1960). The difference in phasic behaviour of the primary and secondary endings, if existing under a wide range of conditions, is likely to be of great functional significance. The phasic sensitivity of the primary ending is somewhat similar to the 'phase advance' or 'velocity feedback' used to stop oscillation in some inanimate control systems, and is thought

to be of similar importance in the reflex stabilization of muscle contraction (Merton, 1951; Pringle & Wilson, 1952; Lippold *et al.* 1958). The secondary ending with so much less phasic sensitivity behaves more like a pure length receptor, though it should be noted that its static response may be altered by intrafusal fibre contraction. It should also be noted, for this might be important for reflex organization, that the passive sensitivity (in impulses/sec/mm) of secondary endings to slowly applied stretch is similar to that of primary endings.

In assessing the results obtained during activation of the intrafusal muscle fibres it should be remembered that the method of activation was artificial, and that different results might be obtained with physiological discharges of motoneurones. During ventral root stimulation, whether of large filaments or those containing only a single γ fibre, the resulting intrafusal fibre contraction might be more asymmetrical (particularly of any muscle fibres only capable of non-propagated contractions) than any that would occur normally, and the excitatory effect exerted on the ending with the muscle at a series of different lengths might be different in the two cases. The finding that increasing the strength of intrafusal fibre contraction (by increasing the frequency of stimulation) may increase the slope of the frequency–extension relation is in agreement with previous work showing that the excitatory effect on the ending of intrafusal fibre contraction may be greater at greater lengths of the muscle (Leksell, 1945; Kuffler *et al.* 1951; Whitteridge, 1959). The contrasting finding that the slope of the frequency–extension relation did not always increase with an increasing excitatory effect on the ending appears, however, to be of equal interest. It may be asked whether any physiological significance should be attached to these types of responses and whether they perhaps depended primarily upon the contraction of the two kinds of intrafusal muscle fibres now suggested by histological work (Boyd, 1959, 1960; Barker & Ip, 1960; Cooper, 1960; Swett & Eldred, 1960). But these and other problems arising out of the present work demand further experimentation for their solution. It is apparent only that the effects of intrafusal fibre contraction on spindle afferent endings cannot all be ascribed to a change in a single parameter of a linear frequency–extension relation.

SUMMARY

1. The discharge of single afferent endings of de-efferented muscle spindles in the soleus muscle of the cat was studied during extension of the muscle at a constant velocity of 2·3–3 mm/sec. The afferent endings were classified as primary (35) or secondary (20) according to the conduction velocities of their afferent fibres.

2. During passive stretch the secondary endings were appreciably less sensitive to the dynamic component of the stretch than were the primary endings, for their frequency of discharge fell much less when the dynamic stretching was stopped and a steady extension maintained. On beginning to release the stretch at a slow rate the primary endings usually stopped firing abruptly, while the secondary endings did not. These differences between the two kinds of ending are probably due to their different anatomical relationship to the intrafusal muscle fibres.

3. In both cases when, for the period of dynamic stretching, the frequency of discharge of the ending was plotted against the extension, an approximately linear relation was usually found. The values of the slope of this frequency–extension relation ranged from 2·5 to 15·3 impulses/sec/mm, and were not consistently different for the two kinds of ending. The secondary endings, however, usually had an appreciably higher initial threshold for extension (or tension). The frequency–extension relations were usually little different when slightly higher or slightly lower rates of stretch were used.

4. Some effects of intrafusal fibre contraction on the frequency–extension relation were investigated by stimulating large ventral root filaments containing both α and γ motor fibres. The strength of the intrafusal fibre contraction was graded, without change in the strength of the extrafusal fibre contraction, by varying the frequency of stimulation from 30 to 160/sec. For both types of ending considerable increases in the slope of the frequency–extension relation were frequently produced by intrafusal fibre contraction. In some cases, however, there was no significant change in slope in spite of an increased excitatory effect on the ending. The reasons for these differing effects of intrafusal fibre contraction are not known.

5. Certain findings suggest that similar results would have been obtained for the frequency–extension relation determined under static conditions.

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THE ACTION OF 5-HYDROXYTRYPTAMINE AND RELATED COMPOUNDS ON NEUROMUSCULAR TRANSMISSION IN THE LOCUST *SCHISTOCERCA GREGARIA*

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(*Received* 3 *March* 1961)

At the present time the nature of the junctional transmitter in insects is not known. Substances such as acetylcholine or 5-hydroxytryptamine, known or suspected to be neurohumours in other groups of animals are not, in general, definitely known to have an action on insect neuromuscular transmission. Harlow (1958) found that acetylcholine (ACh) did not interfere with the response of the extensor tibialis of the hind leg of the locust *Locusta migratoria* to stimulation by the crural nerve, although perfused ACh could produce a tetanic extension of the whole leg. The nature of the action, on a locust neuromuscular junction, of an active amine such as 5-hydroxytryptamine (5-HT) which is generally (Welsh, 1957*a*) although not always (Welsh, 1957*b*) found to have an opposite effect from that of ACh, should be of interest. Twarog & Roeder (1957) found that, although highly active on the heart of the cockroach, 5-HT was effective only at high concentrations on the cercal synapse of the desheathed last abdominal ganglion. Since we too found that 5-HT must be applied in high concentrations, ACh in the same concentrations was applied as a check against non-specific effects. Since tryptamine has been found to be far less active than 5-HT on preparations where 5-HT is thought to be a neurohumour (Hill, 1958; Greenberg, 1960), we also employed tryptamine as a test of the specificity of 5-HT.

Davey (1960) has suggested that an *o*-dihydroxyindolalkylamine may have an excitatory role in the insect peripheral nervous system, and that its action may be inhibited by 5-HT, through a blocking of receptor sites by the structurally similar 5-HT. This suggested to us the use of a dimethoxyindolalkylamine in the search for an antagonist to 5-HT on our preparation. We also tested as possible antagonists lysergic acid diethylamide (LSD) and bromolysergic acid diethylamide (BOL) which are known to act as antagonists to 5-HT in other invertebrate preparations (Welsh, 1957*a*), probably by virtue of irreversibly imitating 5-HT. 3-(pyrrolidinomethyl)-thionaphthene is known to be one hundred times as active as

LSD as an antagonist of 5-HT for the rat uterus preparation (personal communication from Mr J. J. Lewis), and this compound also was tested on our preparations.

METHODS

Activity of the neuromuscular junctions associated with the 'fast' responses of the metathoracic extensor tibialis and flexor tibialis to stimulation of the crural nerve was studied during the application of 5-HT and other compounds, in the locust *Schistocerca gregaria*.

Tension development in a muscle was recorded by an RCA 5734 mechano-electronic transducer, of which the plate shaft was attached to the femur or (for more isometric conditions) directly to the apodeme of the muscle studied. Simultaneously, the resting potential and action potential of a single muscle cell were recorded internally with a glass micro-electrode filled with 3M-KCl solution. The crural nerve was stimulated by supra-maximal square pulses, of variable strength, delay, and duration, through chlorided silver hooks placed on the nerve trunk near the metathoracic ganglion, from which it had been severed. External recording from the crural nerve and from the extensor tibialis was accomplished by similar electrodes.

A double micro-electrode technique similar to that described by del Castillo, Hoyle & Machne (1953) was used to record the membrane response of single muscle cells to internally applied cathodal and anodal pulses, and to measure membrane resistance.

Drugs were applied in solution, in locust saline made according to the formula of Hoyle (1954). Those used were acetylcholine iodide, tryptamine hydrochloride (both Eastman), 5-hydroxytryptamine creatinine sulphate, lysergic acid diethylamide, bromolysergic acid diethylamide, 5,6-dimethoxytryptamine (all Sandoz), and 3-(pyrrolidinomethyl)-thionaphthene (Pharmacology Department, University of Glasgow).

Experiments were carried out in a room thermostatically controlled at 21° C.

RESULTS

Effects of 5-HT and related compounds on the response of muscle fibres to presynaptic stimulation. The normal mechanisms of the neuromuscular junction studied have been established by Hoyle (1955) in the related *Locusta migratoria*. The twitch of the metathoracic extensor tibialis or flexor tibialis resulting from a single supra-maximal shock to the fifth thoracic nerve is accompanied by an electrical response in every innervated fibre. This action potential consists of a large e.p.p.-like response plus a spike which often overshoots the zero potential base line.

Injection into the leg of 10^{-2}M tryptamine or 5-HT reduced the response in surface fibres of the flexor tibialis to an e.p.p. in less than 1 min (Figs. 1*a* and 2*a*). As the spike became reduced its origin from the e.p.p. was progressively delayed. When the drug was washed away by injection of fresh locust saline, recovery to a full spike occurred in less than 1 min (Figs. 1*b* and 2*b*). It may be seen from Fig. 1*a* that tryptamine caused an increase in synaptic delay from which the preparation did not recover (Fig. 1*b*) as rapidly as from the diminution of the spike. 5-HT also produced an increase in synaptic delay.

Immersion for 4 min or more in 10^{-2} M tryptamine or 5-HT completely abolished the electrical response of the surface fibres, but as deeper fibres were affected more slowly the development of tension by the entire flexor tibialis was not abolished until after 30 min or more of soaking in either drug.

With decreased concentrations of 5-HT or tryptamine loss of twitch tension in the extensor tibialis proceeded more slowly (Fig. 3*a*, *b*). After

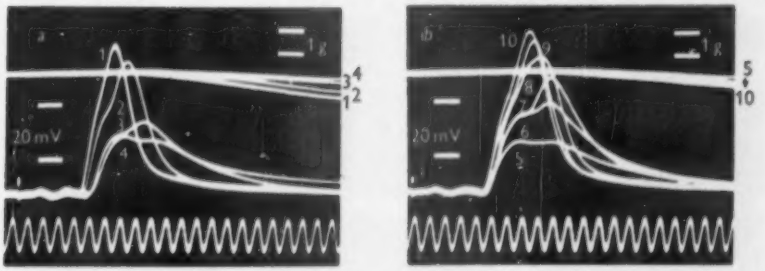

Fig. 1 (*a*) Simultaneous superimposition of tension development (upper trace) and intracellularly recorded action potentials in the flexor tibialis during inhibition by tryptamine. The first trace was photographed in normal locust saline 10 sec before application of 10^{-2} M tryptamine. Succeeding traces were photographed at 5 sec intervals after injection of the tryptamine. Note increase in synaptic delay and progressive delay in appearance of the reduced action potential from the e.p.p. Oscillations in the base line after stimulus artifact are due to impulses in coxal muscles.

(*b*) A similar record of recovery. The fifth trace was photographed in 10^{-2} M tryptamine. Succeeding traces were photographed at 5 sec intervals after injection of fresh normal locust saline. Note that synaptic delay does not recover as rapidly as does return of action potential. Time signal, 500 c/s in both.

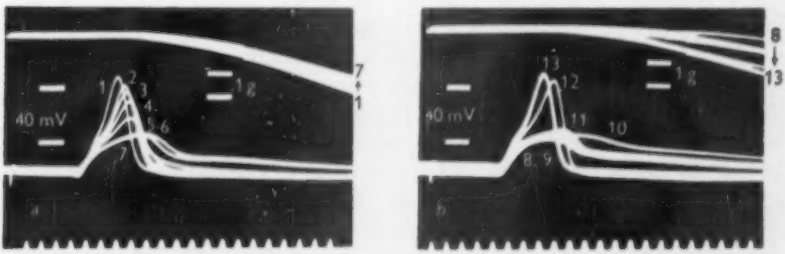

Fig. 2. (*a*) Simultaneous superimposition of tension development (upper trace) and intracellularly recorded action potentials in the flexor tibialis during inhibition by 5-HT. Increase in synaptic delay is not marked in this photograph, because not enough time had elapsed for a return to normal synaptic delay since subjection to tryptamine. Traces superimposed at 5 sec intervals.

(*b*) A similar record of recovery from 5-HT. Traces superimposed at 5 sec intervals. Time signal, 500 c/s in both.

30 min in 10^{-3} M 5-HT twitch tension was reduced by 25 % in most experiments, but could be restored by injection of fresh saline to wash out the 5-HT. Immersion for longer periods led to the recovery on washing with normal locust saline being incomplete, but recovery could be greatly enhanced by using saline containing excess calcium (10 mM) or entirely lacking in magnesium. Complete abolition of the twitch was obtained after 10–15 hr in 10^{-3} M 5-HT. In most preparations there was a 10 % decrease in resting potential during the first hour. The resting potential thereafter remained practically constant at its new value (presumably a

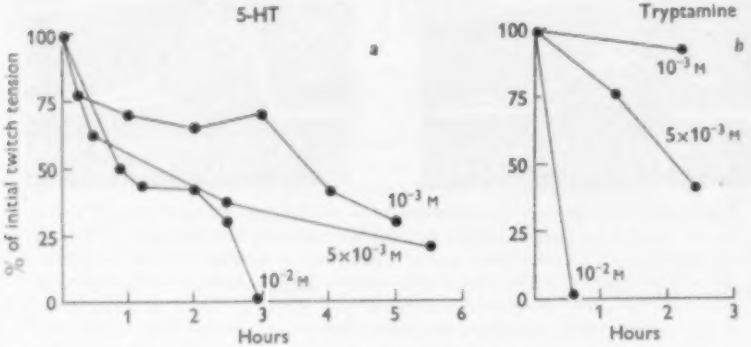

Fig. 3. (*a*) Relation of tension of single twitches of extensor tibialis to time of soaking in 5-HT.

(*b*) Relation of tension of single twitches of extensor tibialis to time of soaking in tryptamine. Numbers show molar concentration.

value reduced by electrode injury). The action potential showed a progressive decline. The initiation of the spike from the e.p.p. was progressively delayed, meanwhile becoming reduced in height. After about 5 hr in 10^{-3} M 5-HT only an e.p.p. type of response remained. This in turn diminished during about 10 hr, until finally it disappeared in all fibres at the time at which no twitch tension could be recorded. At this point the resting potentials of the fibres were still at the value reached after the first hour.

The effect of 10^{-3} M tryptamine on the electrical response of the fibres of the extensor tibialis to nerve stimulation was similar to that of 5-HT, except that there was usually a rise in the height of the action potential during the first 15 min or so, reaching about 10 % and then declining as in the same concentration of 5-HT.

Attempts to find an antagonist to 5-HT for this preparation were unsuccessful. LSD, BOL, 3-(pyrrolidinomethyl)-thionaphthene and 5,6-dimethoxytryptamine all proved to have effects on the mechanical and

electrical responses of the extensor tibialis similar to those of 5-HT or tryptamine, and in similar concentrations.

Effect of acetylcholine on response of muscle fibres to pre-synaptic stimulation. Treating the extensor tibialis with ACh for 10 hr by injection of 10^{-1} M ACh solution had no effect on isometric twitch tension. Action potentials still developed normally after stimulation through supramaximal shocks to the crural nerve.

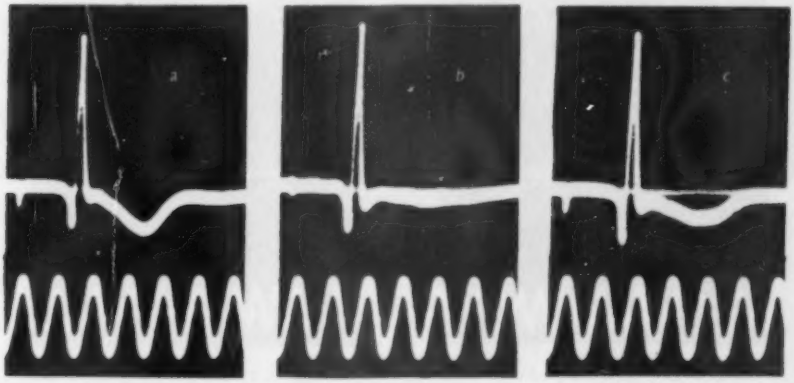

Fig. 4. Recording by external electrodes from the crural nerve trunk of nerve action potential (first deflexion) and muscle action potential (second deflexion): (*a*) in normal locust saline; (*b*) after 1 min in 10^{-3} M tryptamine; (*c*) 1 min after washing with normal locust saline. Time signal, 500 c/s.

Effects of 5-HT and related compounds on the nerve action potential. Recordings were made with external electrodes from the crural nerve trunk to test the possibility that the action reported in the preceding section could be on presynaptic nerve. Neither 5-HT, tryptamine, LSD, BOL, nor 3-(pyrrolidinomethyl)-thionaphthene inhibited the action potentials of the 'fast' nerve fibres supplying the extensor tibialis and flexor tibialis (Fig. 4). Even when all trace of tension development or electrical response in the muscle fibres was lost, the nerve impulse remained maximal.

Effect of 5-HT and tryptamine on active membrane response and on membrane resistance of muscle fibres. When cathodal pulses are applied intracellularly to fibres of the metathoracic flexor tibialis, active membrane responses appear after depolarization exceeds about 12 mV. These responses are oscillatory and resemble those found by del Castillo *et al.* (1953) in muscle fibres of *Locusta migratoria* and by Cerf, Grundfest, Hoyle & McCann (1959) in muscle fibres of *Romalea microptera*. Anodal pulses produce purely electrotonic responses, like those produced by cathodal pulses subthreshold for the active membrane response but of opposite sign.

Neither 10^{-2}M tryptamine (Fig. 5) nor 10^{-2}M 5-HT had any effect on the development of the active membrane response or on the electrotonic response to anodal pulses. Membrane resistance remained constant during immersion for up to 5 hr in 10^{-2}M 5-HT or 10^{-2}M tryptamine.

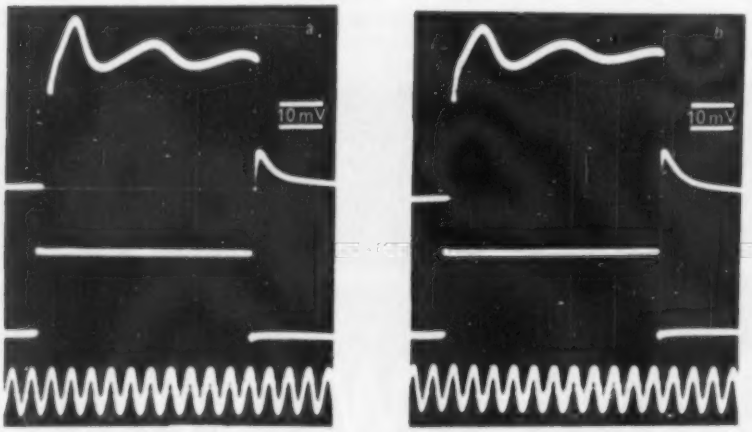

Fig. 5. (*a*) Active membrane response (upper trace) to cathodal stimulus in normal locust saline. Lower trace shows the voltage drop produced by the current flowing through the membrane across a 30 KΩ monitor resistance.

(*b*) Response of the same flexor tibialis fibre after 10 min in 10^{-2}M tryptamine. Change in oscillatory response is no greater than would occur in normal locust saline over same interval since initiation of repetitive stimulation. Time signal, 500 c/s in both.

DISCUSSION

5-HT and related compounds evidently affect synaptic transmission, since it has been shown that the inhibition they produce cannot be due to blockage or transmission in nerve fibres or to diminution of the responsiveness of the electrically excitable component of the muscle-fibre membrane. In fact, single muscle fibres could be seen to twitch when stimulated intracellularly, even after the twitch response of the entire flexor tibialis to stimulation of the fifth thoracic nerve had been abolished by application of high concentrations of 5-HT or of tryptamine. Probably 5-HT-like compounds are either preventing release of transmitter substance from nerve terminals or blocking receptor sites at the junctional regions of the muscle cells.

The high concentrations required may have some relation to the properties of the membranes surrounding insect nerves and neuromuscular junctions. A peripheral nerve axon is directly surrounded by a loose

mantle of membranes derived from the mesaxon of a lemnoblast, until it reaches the region of neuromuscular junctions of a muscle cell. Here the axon runs in a groove, in the surface of the muscle fibre, which is covered by the merged basement membranes of the tracheoblast and the lemnoblast (Edwards, Ruska & de Harven, 1958). Hoyle (1952, 1953) and Twarog & Roeder (1956) have shown that insect nerve sheaths interfere with penetration to the axon of ions and of pharmacological agents.

O'Brien & Fisher (1958) suggest that the toxicity to insects for neuropharmacological compounds may be diminished if insect nerve sheath is relatively impermeable to ionized compounds, thus permitting only the un-ionized fraction to be effective. Similarly, if the membranes covering insect neuromuscular junctions form a barrier to the diffusion of ionized compounds, this might explain why we had to use such high concentrations. All experiments were carried out in locust saline at a pH of 6·9. If the barrier to ionized fractions is assumed to be completely effective, the

TABLE 1

Compound	pKa	Fraction un-ionized at pH 6·9
BOL	5·40	0·97
LSD	5·90	0·91
tryptamine	9·10	0·016
5,6-dimethoxytryptamine	9·12	0·017
5-HT	9·15	0·018

fraction of each compound un-ionized at pH 6·9 will correspond to the effective concentration. In Table 1 (calculated from the formula given by Albert, 1952) pKa values and fractions un-ionized at pH 6·9 are listed for five of the compounds used. From the fraction un-ionized, BOL and LSD should be nearly one hundred times as effective in penetrating as the three other compounds, but in fact there was no marked difference in effectiveness on our preparation. In any case, the effective concentration for 5-HT and tryptamine would still be very high compared to the concentrations effective in other invertebrate preparations. It might be suspected that the inhibition found was merely due to non-specific side effects, if it were not that acetylcholine, in concentrations ten times higher still, is not at all toxic.

All the compounds found to be inhibitory are indole amines or have in common a structural resemblance to indole amines. Greenberg (1960) tested the activity of a large number of tryptamine analogues on the *Venus mercenaria* heart and reported that the simplest structural requirement for 5-HT-like activity was a flat aromatic nucleus with a 2-aminoethyl side chain. This requirement is fulfilled by most of the compounds we found to be effective inhibitors of neuromuscular transmission in the

locust leg. Perhaps they act by blocking the receptor sites for a transmitter with a chemical structure just dissimilar enough to prevent the 5-HT-like compounds substituting for it in excitation.

SUMMARY

1. The effect of tryptamine analogues on the neuromuscular transmission of impulses in the jumping leg of the locust was studied by recording action potentials and active membrane responses with intracellular micro-electrodes, and by recording tension with a mechano-electronic transducer.

2. High concentrations of 5-HT, tryptamine, and a number of tryptamine analogues block the development of an action potential and of tension in fibres of the metathoracic extensor tibialis and flexor tibialis muscle.

3. High concentrations of the same compounds do not block the action potential in the fibres of the crural nerve which supplies 'fast' motor axons to the extensor and flexor tibialis.

4. Tryptamine analogues do not inhibit the active membrane response in fibres of the extensor or flexor tibialis, or alter their membrane resistance.

5. It is concluded that the tryptamine analogues tested block neuromuscular transmission.

We wish to thank Dr Graham Hoyle for advice during the course of our investigations; Mr J. J. Lewis and Dr Martin Smith for discussions on the pharmacological aspects of this paper; Mr S. T. Reid for supplying a sample of 3-(pyrrolidinomethyl)-thionaphthene; and Dr A. Hofman of Sandoz for providing the pKa values listed in Table 1 and for supplying a sample of 5,6-dimethoxytryptamine. This investigation was carried out during the tenure by one of us (R.B.H.) of a post-doctoral fellowship, HF-5211-C2, from the National Heart Institute, United States Public Health Service.

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SENSITIZATION OF THE SUBMAXILLARY AND PAROTID GLANDS BY REMOVAL OF PART OF THE AFFERENT INFLOW TO THE SALIVARY NUCLEI

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During prolonged treatment of cats with parasympatholytic agents the salivary gland cells acquire a supersensitivity to sialogogue drugs. This suggests that the sensitivity of the untreated gland is maintained at a low level through some action of acetylcholine, set free from the endings of the post-ganglionic parasympathetic fibres (Emmelin & Muren, 1950; for discussion see Emmelin, 1952, 1961*a*). The supersensitivity produced by parasympatholytic drugs resembles that caused by cutting the pre-ganglionic parasympathetic fibres, but exceeds it. To explain this it has been assumed that the normal level of sensitivity is determined in part by acetylcholine released by impulses from the central nervous system and partly by acetylcholine leaking from the endings of the post-ganglionic neurone independently of nerve impulses (see Emmelin, 1960, 1961*b*).

In the present experiments an attempt was made to study the dependency of the sensitivity on nerve impulses. The sensitivity of the submaxillary and parotid glands to secretory agents was observed over long periods of time, and the effect of reducing the flow of secretory nerve impulses investigated; for this purpose afferent fibres from the tongue, carrying impulses to the medullary salivary centres, were severed.

A preliminary report of some of these experiments has been given (Emmelin, 1956).

METHODS

Cats were used as experimental animals. The submaxillary glands were stimulated by adrenaline or methacholine, the parotid glands, very little sensitive to adrenaline, by methacholine only. The sensitivity of the glands was estimated once a week, by means of a short-acting barbiturate given intracardially after preliminary ether. When adrenaline was used to evoke secretion, hexobarbitone was injected. This drug, which in our experience is very suitable for these experiments, could not be used when methacholine was the test agent because of its slight parasympatholytic effect; in those cases Baytinal (sodium 5,5-allyl-(2′-methyl-propyl)-thiobarbiturate) was given instead. The atropine-like effect of hexobarbitone was sufficiently pronounced to abolish all reflex secretion; when Baytinal was used this was achieved by injecting hexamethonium 5 mg/kg intracardially.

Fine glass cannulae were inserted temporarily from the mouth into the two submaxillary or parotid ducts of the anaesthetized animals. Standard doses of adrenaline (2, 5 and

10 μg/kg) or methacholine (0·5, 1 and 2 μg/kg) were administered intracardially and the drops of saliva falling from the cannulae were counted.

When the level of sensitivity of the normal glands had been determined, the lingual nerve fibres were cut as far medially as possible to the submaxillary ducts, or the glossopharyngeal nerves near the exit from the skull. In some experiments the chorda-lingual nerve of one side was cut in order to produce a preganglionic parasympathetic denervation of the corresponding submaxillary gland.

RESULTS

Submaxillary glands

Section of the lingual nerves. Figure 1 summarizes observations made in twenty-five cats, using adrenaline to evoke secretory responses from the submaxillary glands. It can be seen that unilateral section of the lingual fibres increased the sensitivity of both submaxillary glands, but particularly that of the operated side. When the lingual fibres of the other side were cut as well there was a further rise in sensitivity bilaterally and eventually the two glands became equally supersensitive. This was the effect seen in most cats. In some animals both glands were sensitized to the same extent by

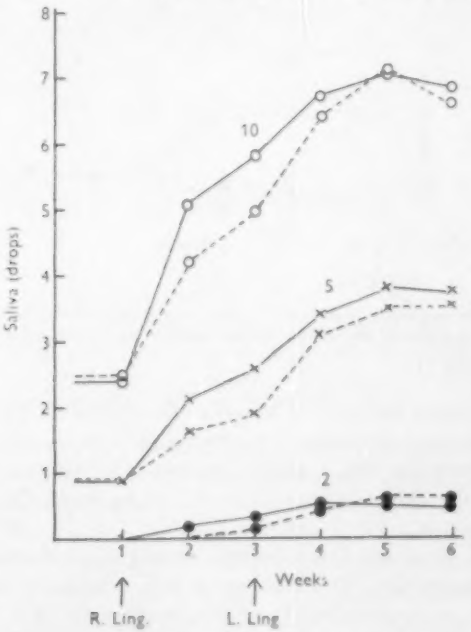

Fig. 1. Average responses of the submaxillary glands to adrenaline in 25 cats. —— right gland, – – – left gland. Responses to adrenaline: ○ 10 μg/kg, × 5 μg/kg, ● 2 μg/kg. At the first arrow the right, at the second the left, lingual fibres were cut.

the unilateral nerve section and when the fibres of the other side were severed the two curves continued to rise together; in some, only the gland of the operated side was sensitized and in one cat neither of the glands was affected until the lingual fibres of both sides had been cut.

The supersensitivity produced by cutting the afferent lingual fibres was less pronounced than that seen after section of the chorda tympani, as will be shown below, but resembled it in the following respects. It developed gradually, first becoming discernible after 2 or 3 days and

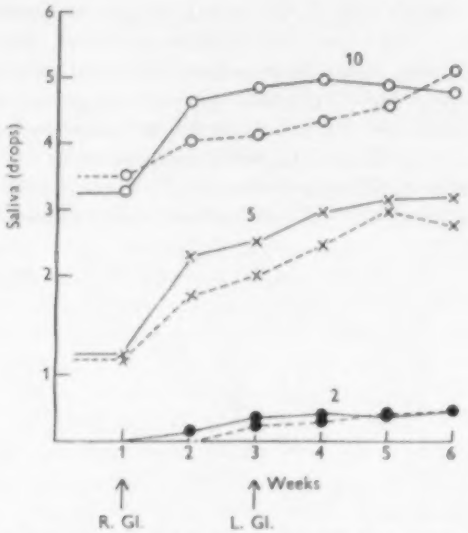

Fig. 2. Average responses of the submaxillary glands to adrenaline in eight cats. At the first arrow the right, at the second the left glossopharyngeal nerve cut. Symbols as in Fig. 1.

reaching a maximum within 2 or 3 weeks. The threshold was lowered and the responses to moderate doses of adrenaline were increased. Furthermore, the sensitization was found to be unspecific, just as that obtained after section of the chorda, as could be seen when methacholine was given to produce secretion.

Section of the glossopharyngeal nerves caused a supersensitivity of the submaxillary glands also. This is shown in Fig. 2, which gives the average responses of eight cats to the three standard doses of adrenaline. The supersensitivity was similar to that caused by cutting the lingual nerves but seemed less marked. Sensitization to methacholine was also obtained.

Section of the chorda tympani. In order to compare quantitatively the

supersensitivity produced by section of the afferent fibres and that brought about by abolishing all impulses from the central nervous system by way of the chorda, the following type of experiment was carried out. The sensitivity of the two submaxillary glands to adrenaline was estimated. The right lingual fibres were then cut; then after 2 weeks the left lingual

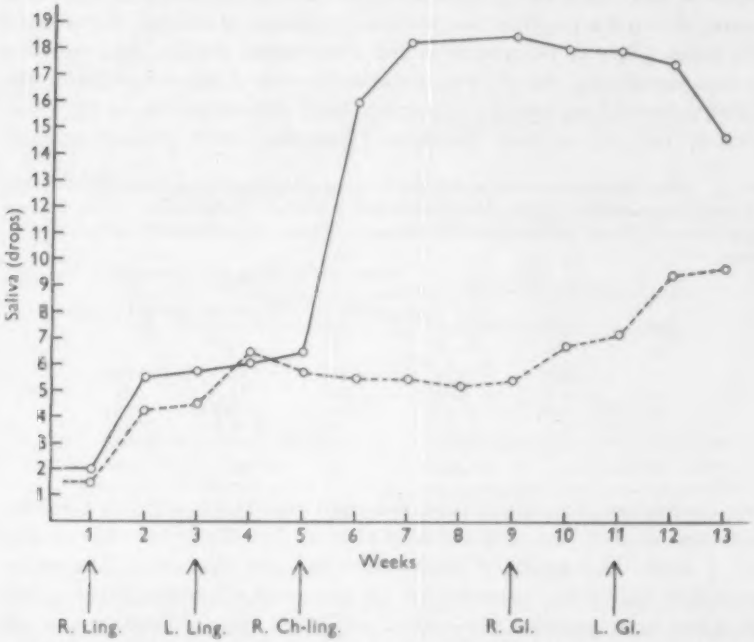

Fig. 3. Secretory responses of the right submaxillary gland (——) and the left gland (– – –) to 10 μg adrenaline/kg, in one cat on thirteen occasions with weekly intervals. The arrows mark, from left to right: section of right lingual, of left lingual fibres, of right chorda-lingual nerve, of right and of left glossopharyngeal nerves.

fibres as well, and 2 weeks later the right chorda-lingual nerve. Supersensitivity due to section of the chorda was left to develop for 4 weeks; the right glossopharyngeal nerve was then severed and 2 weeks later the left nerve. Thus the right gland was deprived of all impulses sent through the chorda and the left gland of all impulses reflected from the four afferent nerves. The level of sensitivity of the two glands was determined weekly. Figure 3 shows such an experiment. It can be seen that supersensitivity created by cutting the glossopharyngeal nerves can be added to that caused by section of the afferent lingual fibres, but not to that produced by section of the chorda. The level of sensitivity attained in the left gland

is considerably lower than that of the right gland. This was the case in five other cats also, which survived all these operations. The cat of Fig. 3 is, in fact, the one in which the difference between the two sides was least marked.

Although these experiments were carried out over a period of about 3 months only, and the precaution was taken to re-cut the fibres first severed, it is quite possible that some regeneration of afferent fibres could have taken place in the course of the observation period, thus reducing the supersensitivity. In order to diminish the risk of this complication the following type of experiment was tried. After the estimation of the sensitivity of the two normal glands to adrenaline both glossopharyngeal

TABLE 1. Drops of saliva secreted in response to 10 μg adrenaline/kg: *A*, before denervation; *B*, 1 week after section of both glossopharyngeal nerves; *C*, 1 week after section of right lingual fibres; *D*, 2 weeks after section of left lingual fibres; *E*, 3 weeks after section of right chorda

	Responses to 10 μg adrenaline/kg				
	Left gland				Right gland
Cat no.	*A*	*B*	*C*	*D*	*E*
1	5·3	8	8·8	10	21·5
2	4·3	11·5	12·5	15	23
3	0·25	1·7	2·5	4·3	12·8
4	0·5	2·7	3·5	7·5	21
5	0·25	2	3	3·7	13
6	2·5	3·5	10·3	13	28

nerves were cut. One week later the right chorda-lingual and 1 further week later the left lingual fibres were severed. The sensitivity was studied once a week, and finally 2 weeks after the last operation. The supersensitivity due to one operation was therefore not allowed to develop fully before the next operation was carried out and it was not possible to study separately the effects of the different operations, as in the series exemplified by Fig. 3. On the other hand, the observation period, 4 weeks, was so brief that regeneration could scarcely obscure the results. Table 1 summarizes the observations made in 6 cats of this series. To make the table easier to read, only the secretory responses to 10 μg adrenaline/kg are given and only the effects on the left gland of afferent, and on the right gland of efferent denervation. The table shows that even with this technique the supersensitivity produced by cutting the four afferent nerves was less pronounced than that caused by section of the chorda.

Parotid glands

Section of the lingual nerves had no definite effect on the sensitivity of the parotid gland to methacholine in six cats examined. In two other cats a slight increase in sensitivity could be observed.

Section of the glossopharyngeal nerves, on the other hand, was found regularly to sensitize the parotid glands to methacholine. This can be seen in Fig. 4, which gives the results of experiments on six cats. When a constant level of sensitivity had been attained in these cats, the right lingual fibres were cut. No further increase in sensitivity ensued, as was the case in the submaxillary glands when glossopharyngeal was added to lingual section.

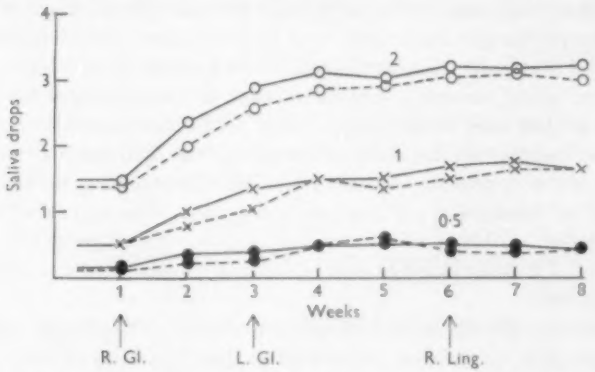

Fig. 4. Average responses of the parotid glands to methacholine in 6 cats. Responses to methacholine: ○ 2 μg/kg, × 1 μg/kg, ● 0·5 μg/kg. —— right parotid gland, – – – left gland. At the first arrow the right glossopharyngeal nerve was cut, at the second the left glossopharyngeal nerve, and at the third the right lingual fibres.

DISCUSSION

The lingual fibres were cut as far medially to the salivary ducts as possible in order not to damage any secretory fibres. This seemed of particular importance since it has been assumed that some secretory fibres for the submaxillary gland, instead of joining the chorda tympani, accompany the lingual nerve for a short distance and then run back to the gland (see Langley, 1898). Injury to such fibres should not affect the contralateral gland. From the facts that unilateral lingual section in most cats sensitized both submaxillary glands and that glossopharyngeal section sensitized the two submaxillary and the two parotid glands it seems legitimate to infer that the supersensitivity was a consequence of severance of afferent fibres.

Numerous investigators have found that the submaxillary gland is particularly affected by electrical stimulation of afferent lingual fibres and the parotid gland by stimulation of afferent glossopharyngeal fibres, and, further, that unilateral stimulation causes the largest secretory responses from the ipsilateral glands (see Miller, 1913; Babkin, 1928). The present

is considerably lower than that of the right gland. This was the case in five other cats also, which survived all these operations. The cat of Fig. 3 is, in fact, the one in which the difference between the two sides was least marked.

Although these experiments were carried out over a period of about 3 months only, and the precaution was taken to re-cut the fibres first severed, it is quite possible that some regeneration of afferent fibres could have taken place in the course of the observation period, thus reducing the supersensitivity. In order to diminish the risk of this complication the following type of experiment was tried. After the estimation of the sensitivity of the two normal glands to adrenaline both glossopharyngeal

TABLE 1. Drops of saliva secreted in response to 10 μg adrenaline/kg: *A*, before denervation; *B*, 1 week after section of both glossopharyngeal nerves; *C*, 1 week after section of right lingual fibres; *D*, 2 weeks after section of left lingual fibres; *E*, 3 weeks after section of right chorda

	Responses to 10 μg adrenaline/kg				
	Left gland				Right gland
Cat no.	*A*	*B*	*C*	*D*	*E*
1	5·3	8	8·8	10	21·5
2	4·3	11·5	12·5	15	23
3	0·25	1·7	2·5	4·3	12·8
4	0·5	2·7	3·5	7·5	21
5	0·25	2	3	3·7	13
6	2·5	3·5	10·3	13	28

nerves were cut. One week later the right chorda-lingual and 1 further week later the left lingual fibres were severed. The sensitivity was studied once a week, and finally 2 weeks after the last operation. The supersensitivity due to one operation was therefore not allowed to develop fully before the next operation was carried out and it was not possible to study separately the effects of the different operations, as in the series exemplified by Fig. 3. On the other hand, the observation period, 4 weeks, was so brief that regeneration could scarcely obscure the results. Table 1 summarizes the observations made in 6 cats of this series. To make the table easier to read, only the secretory responses to 10 μg adrenaline/kg are given and only the effects on the left gland of afferent, and on the right gland of efferent denervation. The table shows that even with this technique the supersensitivity produced by cutting the four afferent nerves was less pronounced than that caused by section of the chorda.

Parotid glands

Section of the lingual nerves had no definite effect on the sensitivity of the parotid gland to methacholine in six cats examined. In two other cats a slight increase in sensitivity could be observed.

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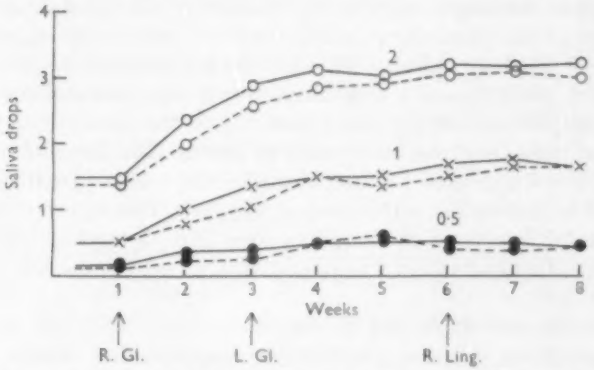

Fig. 4. Average responses of the parotid glands to methacholine in 6 cats. Responses to methacholine: ○ 2 μg/kg, × 1 μg/kg, ● 0·5 μg/kg. —— right parotid gland, – – – left gland. At the first arrow the right glossopharyngeal nerve was cut, at the second the left glossopharyngeal nerve, and at the third the right lingual fibres.

DISCUSSION

The lingual fibres were cut as far medially to the salivary ducts as possible in order not to damage any secretory fibres. This seemed of particular importance since it has been assumed that some secretory fibres for the submaxillary gland, instead of joining the chorda tympani, accompany the lingual nerve for a short distance and then run back to the gland (see Langley, 1898). Injury to such fibres should not affect the contralateral gland. From the facts that unilateral lingual section in most cats sensitized both submaxillary glands and that glossopharyngeal section sensitized the two submaxillary and the two parotid glands it seems legitimate to infer that the supersensitivity was a consequence of severance of afferent fibres.

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observations on the sensitizing effect of cutting the afferent nerves seem to be in agreement with these findings.

In earlier investigations supersensitivity has been produced in various structures by section of efferent nerve fibres or by prolonged administration of drugs interfering with transmission of efferent impulses. In the present experiments the efferent link of the reflex arc was intact. The fact that section of the afferent fibres could produce a supersensitivity of the same type as that caused by cutting the efferent fibres indicates that the sensitivity of the glandular cells is at least to some extent dependent on the flow of secretory impulses from the central nervous system. Cutting one lingual nerve caused a supersensitivity in the submaxillary gland of most cats. The sensitivity could then be further raised, stepwise, by additional section of the other afferent nerves. The level of sensitivity seems to some degree to be determined by the intensity with which the gland cell is bombarded with secretory impulses. This agrees with the fact that repeated injections of a secretory drug like pilocarpine decreases the sensitivity of a denervated or even a normally innervated gland (Emmelin & Muren, 1952).

The supersensitivity caused by bilateral section of the lingual and glossopharyngeal fibres was never as marked as that seen after efferent denervation. It seems reasonable to assume that this was due to the fact that section of the chorda abolished all secretory impulses from the central nervous system, whereas section of the afferent nerves only reduced the flow of impulses. This conclusion is supported by the observation that the first saliva produced by an injection of adrenaline is thick and cloudy when the chorda has been cut in advance, as if no secretion had been going on for some time; after previous bilateral section of the lingual and glossopharyngeal fibres, on the other hand, the first drop of saliva caused by adrenaline is quite clear. The salivary nuclei of the medulla were very likely not completely deprived of afferent supply. It is known, for instance, that secretion of saliva can be elicited reflexly via other nerves than the lingual and glossopharyngeal ones, for instance pharyngeal branches of the vagus.

When preganglionic parasympathetic denervation of the submaxillary gland is intended, usually for practical reasons the chorda-lingual nerve is cut instead of the chorda. The present experiments show that under such conditions the contralateral gland cannot be regarded as an absolutely normal gland, to be used as a control, as is often done. In this connexion it is interesting to note that Strömblad (1956) found unilateral section of the chorda-lingual nerve to reduce the amine oxidase activity not only of the ipsilateral, but probably also of the contralateral submaxillary gland. The role played by afferent denervation for the phenomenon of 'antilytic secretion' will be discussed in a subsequent paper.

SUMMARY

1. The sensitivity of the submaxillary and parotid glands to chemical agents was estimated weekly in cats anaesthetized with short-acting barbiturates.

2. After section of the lingual fibres medially to the submaxillary ducts, or the glossopharyngeal nerves, a supersensitivity was found to develop in the submaxillary gland. Section of the glossopharyngeal nerves sensitized the parotid gland.

3. The supersensitivity resembled that found after section of the chorda tympani but was less pronounced.

4. The findings support the view that the level of sensitivity of the gland cells is in part determined by the intensity of the flow of secretory impulses on the glands.

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J. Physiol. (1961), **157**, *pp.* 410–413
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Printed in Great Britain

ON 'ANTILYTIC' SECRETION OF SALIVA

By N. EMMELIN
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(*Received* 17 *March* 1961)

The phenomenon of paralytic salivary secretion was discovered by Claude Bernard in 1862 and studied by numerous investigators in the second half of the nineteenth century (Bernard, 1862, 1864; Kühne, 1866; Bidder, 1867; Heidenhain, 1868; Langley, 1885; Bradford, 1888). The finding that paralytic secretion is not obtained in animals under chloralose, but is regularly seen when morphine anaesthesia is used, gave rise to the theory that the paralytic flow is caused by catechol amines released by morphine from the adrenals in amounts sufficiently large to cause a secretion from the submaxillary gland cells, sensitized to chemical agents by previous section of the chorda tympani; the early investigators in this field used morphine as an anaesthetic (see Emmelin, 1952, 1961*a*).

When experimenting on unilateral paralytic secretion, Heidenhain (1868) noticed a flow of saliva from the contralateral, normally innervated, submaxillary gland also. It was slower than the flow from the denervated gland. No hypothesis was attempted to explain this phenomenon ('Diese "Sympathie" beider Drüsen ist vorläufig ein völlig unlösbares Räthsel'; Heidenhain, 1883). Langley (1885) observed this secretion also, and coined the term 'antiparalytic' or 'antilytic' secretion.

In order to produce a parasympathetic denervation of the submaxillary gland the chorda-lingual trunk is commonly cut instead of the chorda alone; the secretory fibres leaving the lingual nerve form several strands, and it is considered easier to guarantee a complete section by cutting the lingual nerve before these fibres have branched off. In this operation, however, afferent fibres from the mouth are severed as well and it has been found that unilateral section of the afferent lingual fibres usually causes an increased sensitivity of both submaxillary glands (Emmelin, 1961*b*). Since those investigators who have observed an 'antilytic' secretion have used morphine as an anaesthetic it is tempting to try to explain this phenomenon in the same way as the paralytic secretion; i.e. as due to circulating catechol amines acting on supersensitive gland cells. Section of the afferent fibres produces a much less pronounced supersensitivity than does section of the chorda, which would explain why the 'antilytic' flow is much slower than the paralytic.

The present experiments were carried out in order to see whether unilateral section of the afferent lingual fibres could cause so marked a supersensitivity in the contralateral submaxillary gland that a flow of saliva was obtained when morphine was used as an anaesthetic.

METHODS

The experiments were made on cats. In hexobarbitone anaesthesia the left lingual fibres were cut as far medially to the salivary ducts as possible, as previously described (Emmelin, 1961*b*). Two weeks later the cats were anaesthetized with morphine hydrochloride (25 mg/kg intramuscularly) after preliminary ether and both submaxillary ducts cannulated in the neck. This experiment was carried out in four cats.

Three other cats were treated in the same way with the exception that chloralose (80 mg/kg intravenously) was used as an anaesthetic instead of morphine in the acute experiment. After an observation period of about half an hour the right submaxillary gland was exposed and prepared for perfusion in the following way. All branches of the common carotid artery except that of the gland were tied; similarly, all tributaries of the external jugular vein except one draining the gland were ligated. That cat was heparinized and about half an hour later blood was directed from a cannula in the central part of a carotid artery of a heparinized donor cat, anaesthetized with morphine, through a polythene tube and a cannula into the peripheral part of the carotid of the experimental animal. The blood perfusing the submaxillary gland was not allowed to enter the circulation of the recipient but was collected from a cannula in the jugular vein and returned to the donor through a venous cannula.

RESULTS

In the four cats anaesthetized with morphine some secretion was observed from both submaxillary glands; it resembled the paralytic secretion seen in earlier experiments (Emmelin & Muren, 1950, 1951*a*) in being slightly irregular and tending to decrease in the course of the experiment. In two of the cats the flow was just perceptible, in one of them about one drop was produced in 5 min in the early part of the experiment; the remaining cat secreted about one drop per minute. There was no great difference in secretory rate between the two glands of one and the same animal. In the two best secreting animals both suprarenals were removed. This caused the flow of saliva to cease within about 3 min.

Figure 1 shows the results obtained in one of the perfusion experiments. The two submaxillary glands of the cat anaesthetized with chloralose showed a marked sensitivity to adrenaline, the right gland producing 9 drops and the left one 12 drops in response to 10 μg adrenaline/kg intravenously. When adrenaline was not given there was no flow of saliva. This was the case when the gland had been prepared for perfusion also, but as soon as perfusion with blood from the morphine cat had been established a secretion started, about one drop falling every minute. The flow ceased when 1 mg dihydroergotamine was given intravenously to the morphine cat.

Similar although less pronounced results were obtained in the two other perfusion experiments. In one of these the flow was stopped by dihydroergotamine, in the other by removal of the adrenals of the morphine cat.

In the experiment of Fig. 1 there was no secretion from the normal submaxillary gland of the morphine cat. In one of the other donor cats a very small secretion could be observed in the beginning of the experiment.

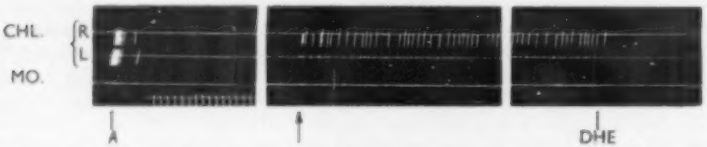

Fig. 1. Secretion of saliva from a submaxillary gland, previously sensitized by section of the contralateral lingual fibres. Records from above downward: secretion from right (R) and left (L) submaxillary glands of a cat under chloralose (CHL.). The left lingual fibres cut 2 weeks earlier. *A*: intravenous injection of 10 μg adrenaline/kg. Line underneath shows no secretion from the left submaxillary gland of a donor cat under morphine (MO.). Time marker, minutes. At the arrow perfusion of the right gland of the chloralose cat with arterial blood from the donor started, and continued throughout the experiment. DHE: intravenous injection of 1 mg dihydroergotamine into the donor cat.

DISCUSSION

In his paper on paralytic secretion reporting also the discovery of the phenomenon 'antilytic' secretion, Heidenhain (1868) mentions that the chorda-lingual nerve of one side had been cut before the acute experiment in morphine anaesthesia. The experiments of the present paper seem to support the hypothesis that the 'antilytic' secretion observed by Heidenhain was the result of two things: (1) There was a marked release of catechol amines from the adrenals; Elliott (1912) showed morphine to have such an effect. (2) The gland cells were supersensitive because of previous section of the contralateral afferent lingual fibres.

Some cases of 'antilytic' secretion have also been described by Langley (1885). Morphine anaesthesia was employed in these experiments. It is not stated that the chorda-lingual nerve was cut; it rather seems as if the chorda itself was severed. If so, the catechol amines released had to act on cells of normal low sensitivity. There are great individual variations in adrenaline sensitivity of submaxillary glands (Emmelin & Muren, 1951*b*). In occasional cats the normal gland may be sensitive enough to secrete when morphine is given, as is seen in the present and in earlier experiments (Emmelin & Muren, 1950). This might explain the observations made by Langley. It is possible, however, that this supposition is erroneous and

that a complete explanation of the phenomenon of 'antilytic' secretion is still lacking.

SUMMARY

1. Two weeks after unilateral section of the lingual fibres medial to the salivary ducts both submaxillary glands of cats under morphine anaesthesia show secretory activity.

2. Under similar conditions no secretion is obtained if chloralose is used as an anaesthetic instead of morphine; but if the gland is then perfused with blood from a cat under morphine anaesthesia, secretion starts.

3. These experiments throw some light on the phenomenon of 'antilytic' secretion.

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PROCEEDINGS OF
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BRISTOL MEETING

14–15 *April* 1961

Variable and free mucopolysaccharides in the rat's kidney

By J. M. N. Boss, P. Breddy and G. F. Cooper. *Department of Physiology, University of Bristol*

During the course of investigations into the effect of water diuresis and anti-diuretic hormone on renal histology in the rat (Breddy, Cooper & Boss, unpublished observations), the distribution of mucopolysaccharides by means of the periodic acid-Schiff reaction was examined.

Brush borders. We have confirmed the finding of Longley & Fisher (1954), that the brush border stains more deeply by the P.A.S. reaction in the second part of the convoluted tubule than in the first. We were unable to detect any effect of diuresis or hormone on the appearance of the brush border in either segment.

Intercellular cement. P.A.S.-positive material is present between some cells of the collecting ducts, but may be absent or scanty in all or part of a cross-section of one of these tubules. Such material between cells of the collecting ducts appears linear in optical section, but if an intercellular boundary is in a plane to which the line of vision is approximately vertical, the P.A.S.-positive material between the cells is found to be granular. The granules vary in size up to about 1 μ in diameter, are densest near the basement membrane, and vary in number between tubules. These variations are related neither to water diuresis nor to its inhibition by anti-diuretic hormone.

Surface layer. A periodic-acid-Schiff (P.A.S.)-positive layer, less than 0·5 μ thick, covers the luminal surface of the distal convoluted tubules. A similar layer is found in the collecting ducts, but is inconstant.

Free mucopolysaccharide. The lumen of a collecting duct may contain P.A.S.-positive material. This varies in amount from small granules on the free surface of the cells, suggesting irregular swelling of the cuticle, to large masses free in the lumen.

Plasma mucopolysaccharide. Some medullary capillaries contain P.A.S.-positive plasma.

These results indicate that P.A.S.-positive material, presumably acid

mucopolysaccharides, may pass into the collecting ducts and medullary capillaries of the kidney.

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The removal of rat retinae for metabolic studies

By R. ST J. BUXTON and D. R. COLES. *Departments of Physiology and Medicine, University of Bristol*

Metabolic studies can only be made on fresh tissues. Such experiments require the rapid removal of the tissue from the body into a suitable medium. The present method is a modification of Graymore's (1958) technique and results in a whole retina without choroid being available in a very short time for use in a Warburg apparatus.

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A cannulated crab muscle fibre

By P. C. CALDWELL and G. E. WALSTER. *Department of Zoology, University of Bristol, Laboratory of the Marine Biological Association, Plymouth and Plymouth Technical College*

The leg muscles of the spider crab, *Maia squinado*, contain structures which appear to be fibres roughly 1–2 mm in diameter and up to 3 cm in length (Caldwell, 1958). It has now been found possible to cannulate individual fibres in a manner similar to that used for squid giant axons (Hodgkin & Huxley, 1945) and to carry out membrane potential measurements with an electrode inserted longitudinally and micro-injection experiments. After dissection of a single fibre from the muscle the fibre is cut away from the shell and cannulated at the cut end, the attachment to the apodeme being left intact. Deterioration of the fibre at the cut end tied to the cannula is prevented by the application of a mixture of liquid paraffin and petroleum jelly to the injured region. The effects of the micro-injection of a number of substances with the Hodgkin & Keynes (1956) micro-injector have been studied and Ca, Ba, Sr and caffeine have been found to cause contraction, but not Mg. External application of caffeine, as in the case of frog sartorius muscle (Axelsson & Thesleff, 1958), causes a contraction which does not appear to be mediated by a depolarization of the membrane. In recent experiments cannulated fibres have first been depolarized with solutions of high potassium content and after relaxation have

been reimmersed in crab saline containing 5 mM caffeine, when both contraction of the fibre and repolarization of the fibre membrane took place simultaneously.

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Variations in oxygen availability (O_2a), blood flow and cortical excitability in the human brain

By R. Cooper, H. J. Crow and W. G. Walter. *Burden Neurological Institute, Bristol*

Surgical implantation of up to sixty-eight fine gold electrodes in the brains of epileptic and psychoneurotic patients for investigation and treatment is now an established practice. The electrodes are bared for about 4 mm at the tip and are 150 μ in diameter. They are arranged in ten sheaves inserted through a special introducer held in a stereotaxic frame. They can be left in place for several months.

As well as providing for local recording, stimulating and polarization, the noble-metal electrodes can be used polarographically for indicating the local oxygen availability, as described by Meyer & Portnoy (1959) and others.

Observations of O_2a have been made in many regions of the brain in several human subjects in a variety of conditions, and these have been related to local temperature and blood-flow changes, indicated by variations in resistance of micro-thermistors (250 μ diameter) inserted at the same time. For indicating blood flow the thermistors are heated to a fraction of a centigrade degree above blood temperature.

Records show fluctuations of O_2a with each heart beat and local 'spontaneous' rhythmic variations at about 6/min. The latter are reduced during sensory stimulation, CO_2 inhalation and sleep, increased by O_2 inhalation. Closely adjacent cortical regions show independent variations.

Electrical stimulation to identify functional location of electrodes provides measures of cortical excitability characteristics in terms of stimulus amplitude, duration, frequency and train duration. With a constant-voltage stimulator the 'rheobase' is about 4 V and 'chronaxie' about 80 μ sec at the optimal frequency of 70 pulses/sec. Subjective sensation, local after-discharge and local rise in O_2a have been used as criteria of response. When a sustained after-discharge is evoked the O_2a shows a protracted second rise during which cortical excitability is reduced and local blood flow increased.

The relation between excitability, response, O_2 a and blood flow can be explained in terms of local control of cerebral vascular tone by variations in CO_2 production.

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The use of Barrier Grid storage tubes type 9511A for extraction of average evoked responses from the e.e.g.

By R. COOPER and W. J. WARREN. *Burden Neurological Institute, Bristol*

Dawson (1951) has shown the value of averaging techniques in the study of cerebral responses evoked by various stimuli in human subjects, but his method is limited in resolution and flexibility. Buller & Styles (1959) have demonstrated the application of the Barrier Grid storage tube to the detection of small short-latency responses such as peripheral nerve action potentials in man.

The present system was developed for general application to the analysis of specific and non-specific responses to visual and auditory stimuli in human subjects, including patients with suspected organic brain lesions. Since the after-effects of stimulation may last several seconds, the recording sample duration is adjustable from 100 msec to 2·0 sec. One or more stimuli can be delivered at any time during the recording period in synchrony with the time base. This permits comparison of the averages of events preceding the stimulus with those succeeding it. Six, twelve, twenty-four or forty-eight samples can be accumulated and stored at any desired interval for averaging. The stored information shows no deterioration for several days.

The very low scanning speed at longer sample durations demands extremely low effective beam currents. This is achieved by pulse modulation of the scanning electron beam. Pulses of 1 μsec are generated and the effective beam current is controlled by altering the pulse repetition frequency from 500 c/s to 500 kc/s according to the required sample duration and number.

The averaged outputs from the tubes are written out directly by an Offner type T Transistor Dynograph with the original records. Duplicate tubes permit simultaneous analysis from two regions or second-order averaging.

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The measurement of total gastric blood flow and its relationship to gastric acid secretion in the anaesthetized dog

By J. D. Cumming, A. L. Haigh, E. H. L. Harries and Marjorie E. Nutt. *Department of Physiology, University of Birmingham*

A method has been described for the simultaneous measurement of total gastric blood flow and secretion in the anaesthetized dog (Cumming, Haigh & Harries, 1960).

This method necessitated the removal of the small intestine and a partial colectomy. It was felt that if a method could be devised whereby the bowels were left intact a preparation more approaching the physiological state would result. This has now been achieved.

Dogs are anaesthetized with sodium pentobarbitone I.V. After opening the abdomen the spleen is removed and the omentum is dissected free from the greater curvature of the stomach, care being taken to preserve the chain of short gastric vessels. Partial pancreatectomy is performed to enable the whole length of the splenic vein to be freed from connective tissue. A ligature is placed around the pylorus to occlude the right gastro-epiploic vein. The splenic vein is ligated at its junction with the superior mesenteric vein, and cannulae are inserted both proximally and distally into its length. It is thus possible to collect the entire gastric venous effluent. Post-operative dye injections confirm that the venous drainage is from the stomach only. Blood flow is measured by means of a bubble flowmeter, in a closed circuit, and the blood is returned to a femoral vein.

Under stable control conditions the mean rate of gastric blood flow for thirteen animals was 13·5 ml./100 g stomach (wet wt.)/min S.D. 4·0.

Intravenous infusion of histamine acid phosphate (2·5–10 μg/kg body wt./min) had little immediate effect upon the rate of blood flow, but during the phase of acid secretion the rate of flow increased.

An infusion of adrenaline (1·0–2·0 μg/kg/min), given together with histamine during the phase of histamine-induced acid secretion, resulted in an increase in rate of blood flow with little change in secretory rate.

When noradrenaline was used in place of adrenaline, in the same dosage, a decrease in blood flow occurred together with an inhibition of the acid secretion.

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The essential role of SH and S–S groups in the anaphylactic reaction

By K. A. P. Edman and J. L. Mongar. *Department of Pharmacology, University College London.*

The high activity of sulphydryl (SH) blocking agents in inhibiting the release of histamine by antigen from chopped sensitized guinea-pig lung has led us to investigate the role of SH groups and S–S bonds in the mechanism of the anaphylactic reaction. Both appear to be essential. A 0·1 mM solution of the specific SH blocking reagent *N*-ethylmaleimide reduced histamine release to 44 % of the control value and a 1·0 mM solution to 7 %. Substances which reduce S–S bonds, such as cysteine, thioglycolate and glutathione, potentiated histamine release to an average of 117 % at 10 mM but reduced it to 25 % at 50 mM. These findings suggest that an increase of tissue SH groups through disruption of S–S bonds produces potentiation, whilst an excessive disruption of S–S bonds produces inhibition. The inhibitory effects of S–S reducing compounds were completely reversed by washing in Tyrode solution. Control experiments showed that the inhibition of histamine release was not due to increased tonicity or ionic strength.

Previous work (Mongar & Schild, 1957) indicated that the cellular anaphylactic reaction involves an enzyme system which is inactivated by incubation at 43–45° C but activated at 41–42° C. It has now been found that typical protein denaturing agents such as urea and alcohol also have a dual action on the anaphylactic mechanism. If sensitized lung is preincubated with these agents histamine release by antigen is potentiated at a certain concentration but at higher concentrations it is inhibited. This dual effect may also be due to freeing of SH groups (known to occur in protein denaturation) followed by disruption of the protein structure.

Oxygen lack inhibits histamine release in anaphylaxis (Parrot, 1942). In order to obtain the inhibition it is necessary to reduce oxygen to a low level. Pure nitrogen (O_2 content less than 0·05 %) reduced histamine release to 8 % of the control value but a mixture of nitrogen with 0·5 % oxygen reduced it to only 50 %. The effects of cysteine and oxygen lack are additive. When threshold inhibitory concentrations of cysteine and oxygen were administered together they produced a strong inhibition, suggesting that oxygen lack may also act by way of a reduction of S–S bonds.

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The distribution of sodium in the guinea-pig's skin

By R. St J. Buxton* and E. M. Sedgwick. *Department of Physiology, University of Bristol*

Feldberg & Miles (1953) showed that the skin histamine concentration in the guinea-pig differed in various parts of the body. Miles (Feldberg & Miles, 1953) also found that the skin histamine level was raised after protein sensitization. *In vitro* experiments with the guinea-pig uterus suggested that the tissue sensitivity to histamine was related under certain conditions to the sodium concentration of its environment (McDowall & Soliman, 1953). The following studies were designed to see whether there was any change in skin sodium content under conditions expected to alter the skin histamine level. The hair was closely clipped and whole-thickness samples of skin from the abdomen, buttock, forelimb and cheek were found to have sodium concentrations which were highest in the abdomen and lowest in the forelimb with a mean difference of 3·2 m-equiv/kg of fresh tissue. The potassium level was highest in the cheek, followed by abdomen, forelimb and buttock. The chloride values were not significantly different in the four areas. The differences in sodium content could not be accounted for by the retention of blood in the skin, which was measured in the form of reduced alkaline haematin. The pattern of the sodium content at the four sites examined was not altered by electrolyte depletion with intraperitoneal glucose in 5% solution, nor by sodium loading with saturated sodium chloride given intraperitoneally.

Sensitization with horse serum lowered the sodium content in the skin without changing the pattern, and it was also found that the histamine liberator 48/80 and the antihistamine promethazine reduced the level of sodium and potassium in the skin. The skin water content was reduced after sensitization, 48/80 and promethazine. In spite of the lowering of sodium and potassium levels the chloride content of the skin was raised by 48/80 and promethazine.

The effect of histamine injection, 100 μg subcutaneously, was to decrease the sodium and water levels in the skin. The relationship of skin histamine and sodium concentration after 48/80 was found to be inverse and significant at the 5% level.

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The portal venous concentrations of adrenaline and noradrenaline in patients with portal hypertension

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The amounts of adrenaline (A) and noradrenaline (NA) in human portal-vein plasma have been estimated in two groups of surgical patients. Group 1 consisted of eighteen patients with cirrhosis of the liver undergoing portocaval anastomosis for the relief of portal venous hypertension. Group 2 consisted of nine control patients, with normal portal circulations and livers, undergoing either cholecystectomy for gall stones (eight cases) or vagotomy for duodenal ulceration (one case).

Blood samples were taken at standardized times during the operations. The A and NA were separated by ascending paper chromatography using a water saturated phenol solvent. The eluates, whose R_F values corresponded to those of A or NA were assayed on the rat uterus and the pithed rat blood pressure preparations.

The mean A content in the portal plasma of the nine control patients was 0·24 μg/100 ml. (range 0·01–0·53) and the mean NA content was 0·15 μg/100 ml. (range 0–0·6). In 72 % of the patients with portal hypertension, however, from five to forty times as much A and NA were found to be present in the portal plasma.

In order to localize the anatomical site of origin of the increased A and NA found in the portal plasma, further estimations of these hormones were performed in another group of patients with portal hypertension. Samples of blood were obtained from the portal vein, a jejunal vein, the inferior vena cava cephalad to the renal veins, the abdominal aorta and from superficial arm veins. The regional vascular concentrations of A and NA showed that the increased amounts of these substances were only to be found in the portal venous plasma.

From these investigations it is suggested that the increased amounts of A and NA found in the portal plasma of the majority of patients with portal hypertension are being liberated from the post-ganglionic sympathetic nerve endings in the splanchnic vascular bed.

Artificially induced ovulation in the rabbit

By A. Carlyle and T. D. Williams. *Department of Physiology, University of Bristol*

Hammond (1925) stated that the unmated rabbit is always in a condition of oestrus, providing nutritional conditions are favourable. Ovulation, naturally induced by the act of mating, has been artificially induced by

mechanical stimulation of the anterior vagina (Bishop, 1933; Sawyer, 1949) or by electrical means (Marshall & Verney, 1936).

The present experiments began as a check on the effectiveness of such stimulation. Unanaesthetized mature virgin animals, which had been maintained in individual cages and isolated from the buck, were stimulated for 60 sec with 19 V/10 msec square-wave pulses at 8/sec, by means of bipolar electrodes inserted at least 15 cm into the vaginal canal. An inspection of the ovary was made at laparotomy 24 hr later.

Of an initial series of sixteen animals only one ovulated, although apparently well developed follicles were present. Of a further six animals pretreated with stilboestrol or pregnant mare serum, and showing marked oestrogenic stimulation, only one ovulated. Yet three apparently similar animals, under identical management, accepted the male and became pregnant.

These results suggested the need for a reliable index of oestrus status. Cyclical changes in the vaginal smear pattern have been described by Hamilton (1951) and correlated with different levels of circulating oestrogen; changes in the appearance of the vaginal orifice were considered of limited reliability as a guide to oestrus status (Kunde & Proud, 1929; Friedman, 1938). We have been quite unable to recognize, in the mated or unmated animal, smear patterns similar to those described by Hamilton. We have found, in the unmated animal, well marked changes in the appearance of the vaginal orifice as displayed by light digital traction. The orifice may appear small, pale, crenated and tight '−ve'), or it may be large, deep red to bluish, smooth and relaxed '+ve'). All intermediate states were observed and the appearance might change irregularly and within a few hours.

Using animals showing the two extreme vaginal states we obtained a consistent pattern of responses to electrical stimulation, ovulation being induced in none of the seven animals with a '−ve' vagina and in all of six animals with a '+ve' vagina. We failed to induce it, however, in any of six animals with a '+ve' vagina which were stimulated while under light pentobarbitone anaesthesia. Simple mechanical stimulation in the unanaesthetized animal with '+ve' vagina only resulted in one ovulation out of six.

These results suggest the existence of significant variations in the responsiveness to artificial ovulation-inducing stimuli, while the influence of anaesthesia may be of significance in evaluations of the role of central nervous factors in the ovulation mechanism.

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The influence of light on the occurrence of ovulation in the rat

By A. CARLYLE and SHEILA B. CARTER. *Department of Physiology, University of Bristol*

There is evidence that in the rat ovulation, although spontaneous, is influenced by environmental factors. The present experiments were carried out to confirm and extend the findings of Everett (1948) that in rats under artificial lighting conditions ovulation occurred most commonly between 3½ and 5 hr before the onset of light.

Virgin rats were exposed to alternating periods of 14 hr of light and 10 hr of darkness. Later, darkness was replaced by a dim green light, which was equally effective. The onset of light, whatever its time, is referred to here as dawn, and the 24 hr period following it is considered as a day. Vaginal smears were taken daily and only those rats which had shown four or more regular 4-day oestrous cycles were used. The animals were examined for ovulation around the dawn following pro-oestrus, provided that they showed an oestrous smear. Ovulation was determined by direct inspection of one excised oviduct.

The proportion of rats found at various times to have ovulated were: 7 out of 38 between 2 and 3½ hr before dawn, 21 out of 41 between 2 hr before dawn and ½ hr after dawn and 38 out of 40 between ½ and 2½ hr after dawn.

The time of dawn was then retarded by 8 hr. Twenty days afterwards, the same time relationship between dawn and ovulation was found. None of 10 rats had ovulated 2–3½ hr before dawn while 9 out of 10 had ova in the oviduct 1½–2 hr after dawn.

To test whether an immediately preceding alternation of light and dark was necessary for ovulation, or whether a rhythm, once established, could be maintained without this stimulus, rats which had become accustomed to a diurnal light variation were put under constant light for periods of up to 4 days before an expected day of oestrus. They were then examined at a time when the majority might be expected to have ovulated had the alternations of light and darkness been maintained. The proportions of rats which had ovulated at this time, after 1, 2, 3, and 4 days of constant light, were, respectively 14 out of 14, 11 out of 14, 9 out of 13 and 1 out of 14. Of the 13 rats in the last group which had not ovulated 10 were re-examined 12 hr later. All but one of them had still shed no ova.

On the other hand constant dim green illumination for 4 days did not prevent ovulation occurring by the normal time in 10 out of 11 rats.

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Recordings of electrical activity in the hypothalamus of the rabbit, synchronous with stimulation of the uterine cervix.

By SHEILA B. CARTER and T. D. WILLIAMS. *Department of Physiology, University of Bristol*

Some properties of ankle extensors in the sheep

By K. KRNJEVIĆ and J. F. MITCHELL. *Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge*

Isometric twitch and tetanic responses of the various muscles have been recorded with a torsion-bar myograph. When these contractions are compared with those observed in studies of corresponding muscles of the cat, certain striking differences are immediately apparent.

For instance, judging by the time to the crest of the twitch, even the fastest muscles are much slower (35–45 msec) than the fastest cat muscles at a similar temperature (20–30 msec, Buller, Eccles & Eccles, 1960*b*). The characteristic slow muscles are plantaris and flexor hallucis longus, both of which are typical fast muscles in cats (Buller *et al.* 1960*a*, *b*). Both muscles have very long tendons (cf. Denny-Brown, 1929), inserted in the 2nd and 3rd phalanges, respectively. They clearly play an essential role in maintaining the upright posture ('on tip-toes') in the sheep and their slowness may be considered a useful adaptation. It is perhaps significant that the twitches of these muscles are somewhat complex, with a comparatively fast initial phase (crest-time of 70 msec) followed by a plateau or secondary hump, with a very slow rate of decay (half-decay time of 100–200 msec, compared with 40–50 msec for the fast medial and lateral gastrocnemius, flexor digitorum longus and tibialis posterior, and about 70 msec for slow *cat* muscle.)

Soleus in the sheep is an insignificant bundle inserted into the lateral gastrocnemius. Its total weight is less than 1 g and it develops a maximal tetanic tension of only about 100 g. Yet soleus, whose functional importance in bearing the weight of a 20 kg sheep must be negligible, has all the properties of a typical slow muscle; in fact, it is much slower in its crest-time (120 msec) than either plantaris or flexor hallucis longus.

The distribution of fast and slow muscles among the sheep ankle extensors thus suggests that some muscles have become slow as an adapta-

tion to prolonged weight-bearing, whilst others may be slow quite independently of this function. possibly because of their nervous connexions (cf. Buller *et al.* 1960*b*).

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A search for pharmacologically active substances in fluid from the cerebral ventricles.

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The appearance of pharmacologically active substances in cerebrospinal fluid or in perfusates from the cerebral ventricles has a bearing on several physiological problems, such as the nature of both central synaptic transmitters and substances responsible for functional vasodilatation in brain, and the possible release of hormones of the hypothalamo-hypophysial system into the third ventricle. Various authors have described the presence in these fluids of acetylcholine, catecholamines, 5-hydroxytryptamine (5-HT), histamine, oxytocin, anti-diuretic hormone (AHD) and a kinin-forming enzyme, in physiological and pathological conditions (see Schain, 1960).

We perfused the ventricular system of cats anaesthetized with choralose or pentobarbitone, and examined the effluent from aqueduct or cisterna, before and after stimulation of the brain. The method of perfusion was that described by Bhattacharya & Feldberg (1957). Strong electrical stimuli were delivered through electrodes implanted in the hypothalamus or mid-brain, or with external electrodes at occiput and maxilla. Stimulation produced diffuse autonomic and somatic motor effects. On cessation of stimulation, myoclonic seizures usually occurred.

Acetylcholine appeared in the effluent when the perfusion fluid contained eserine, but there was no increase after activation of the brain. Neither 5-HT nor catecholamines appeared in the effluent, even after intravenous injection of the mono-amine oxidase inhibitor phenylisopropylhydrazine (JB-516, 5 mg/kg), or of reserpine (2 mg/kg). If in addition to JB-516, DOPA (30 mg/kg) was injected in an attempt to raise the noradrenaline content of the brain, just detectable amounts of noradrenaline appeared. Activation of the brain caused no 5-HT or adrenaline to appear in the effluent, neither did noradrenaline increase. Histamine, oxytocin, substance P and ADH were not found, either before or after stimulation;

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neither was the kinin-forming enzyme which according to Chapman, Ramos, Corrado, Fortes & Symmes (1960) appears after stimulation. These results are summarized in Table 1.

TABLE 1. Assay of effluent from cerebral ventricles, perfused at a rate of 0·1 ml./min, on various preparations. When no effect was obtained the result is given as < the smallest amount per ml. effluent that could be detected

Substances	No. of expts.	Leech muscle	Rat uterus (oestrus)	Rat colon	Rat blood pressure	Guinea-pig ileum	Rat urine flow
Acetylcholine	5	*5–20 ng/ml.**	—	—	—	—	—
Adrenaline	10	—	< 5 ng/ml.	—	—	—	—
Noradrenaline	6	—	—	< 20 ng/ml.	*15 ng/ml.†*	—	—
5-Hydroxy-tryptamine	10	—	< 5 ng/ml.	—	—	—	—
Histamine	2	—	—	—	—	< 5 ng/ml.	—
Oxytocin	10	—	< 1 m-u./ml.	—	—	—	—
Substance P	2	—	—	—	—	< 5 u./ml.	—
ADH	1	—	—	—	—	—	< 0·1 m-u./ml.
Kinin-forming enzyme	4	—	None	—	—	—	—

* With eserine in perfusion fluid.
† After intravenous JB-516 and DOPA.

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Effect of perfusion time on the rat heart Langendorff preparation

By J. A. BARCLAY, E. J. HAMLEY and K. WHITE. *Physiology Department, University of Birmingham*

Rat hearts perfused with oxygenated Ringer–Locke solution by Langendorff's method at 80–85 cm solution pressure, 26–28° C and having pH 7·2–7·4 show progressive changes of ionic concentrations in both auricles and ventricles as the duration of perfusion increases. The extent of these changes is greater in auricles than ventricles, the right auricle being most affected and the left ventricle least.

When the total preparation time (from killing the rat by concussion to full perfusion) was kept between 100 and 120 sec, there was an immediate fall of 30 % in tissue sodium in the right auricle in first 2 min of perfusion which continued to 50 % of unperfused (freshly killed) heart values by 15 min. Subsequently sodium increased gradually to 80 % above unperfused, in 1 hr, and in the next hour decreased toward unperfused values. The tissue potassium showed no similar behaviour but after an initial decrease of 20 % in 5 min slowly drifted irregularly downward a further 10 % by 2 hr. The water uptake was immediate but small. This

reached 10% above unperfused in 5 min and thereafter remained about 6% above unperfused values.

From the gradual movements of potassium and water the movement of sodium cannot be ascribed to diffusion of isotonic saline. Nor, in the earlier stages of perfusion, can sodium and potassium be considered to move in equal and opposite directions between tissues and perfusion fluid. The values indicate that any reciprocal movement may be characteristic of only the later stages of perfusion, a condition that may be established by 2 hr and which is maintained thereafter until the heart stops beating.

A general characteristic of the preparation under the conditions of these experiments was that initial perfusion rate was high and was maintained well for the first hour, thereafter the perfusion rate decreased (by 50% at 2 hr). The heart rate tended to decrease by 20–40% at 45 min but changed less rapidly as the preparation aged. In spite of this deterioration in activity the sodium, potassium and water values at 45 min were similar to those at 2 hr. Thus it is improbable that perfusion rate was the controlling factor. It is reasonable to consider that anoxia was greatest during the time of mounting and after 1 hr when perfusion rates decline. Since sodium reached a minimum at 15 min and a maximum at 45–60 min anoxia cannot be the controlling factor. The initial changes in tissue ionic and water contents appeared to be the consequence of perfusion and the pattern of behaviour in the movement of ions represented the attempt by the tissue to adjust to this perfusate.

The effect of hypoxia on guinea-pig bone marrow

By D. J. Moffatt, C. Rosse, I. H. Sutherland and J. M. Yoffey.
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Very few quantitative investigations of bone marrow cells have been carried out in erythroid hyperplasia and hypoplasia.

In the present work, guinea-pigs have been submitted for 5 days to hypoxia (termed 'primary hypoxia') in a decompression chamber, taken out for 5 days ('rebound') and then resubmitted to hypoxia for a further 5 days (termed 'secondary hypoxia'). At the end of each day of the experiment five animals were killed and their bone marrow studied by the quantitative technique of Yoffey (1956). In all, thirty-three control animals and seventy-five experimental animals were used.

The decompression chamber has already been described (Gall, Rogers & Yoffey 1954). The animals were kept at a simulated altitude of 14,000 ft. (4·27 km) and the room temperature was maintained at between 65 and 70° F (18·3–21·1° C). The mean pO_2 was 95 mm Hg.

The total nucleated cell count rose to a peak after 2 days of primary hypoxia and then fell to control level again; in rebound, after an initial rise, it fell steadily until it was well below control level. In secondary hypoxia it reached a high level after 2 days, fell slightly and was rising again at the end of the experimental period.

Nucleated erythroid cells rose in primary hypoxia until they were about 50 % above control level, fell in rebound to about 40 % of control level and rose in secondary hypoxia to about 50 % above control level.

Marrow lymphocytes fell throughout primary hypoxia to about 50 % of control level, rose again in rebound to control level and rose in the first 2 days of secondary hypoxia to about double the control level. They then fell slightly and were rising again by the end of the experiment, by which time they were again nearly 100 % above the control level.

The blood picture during the experiment was of polycythaemia by the end of the phase of primary hypoxia, reduced red-cell count, but still above control, by the end of rebound and polycythaemia again by the end of secondary hypoxia.

Granulocytes in the blood and bone marrow showed no changes during the experimental period.

Blood reticulocytes rose spectacularly in primary hypoxia, fell in rebound and rose again in secondary hypoxia. However, marrow reticulocytes did not rise much above control level in primary or secondary hypoxia though they fell in rebound.

Attempts have been made to correlate the changes in the blood with the changes in the bone marrow in each of the three phases of the experiment. Calculations of stem cell requirements in control and experimental animals have been made.

It is concluded that the lymphocyte has an important role in erythroid hyperplasia.

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Developmental changes in human foetal blood

By D. B. Thomas and J. M. Yoffey. *Department of Anatomy, University of Bristol*

During the first half of gestation the haemoglobin concentration of foetal blood increases rapidly, rising from 4 g/100 ml. at 10 weeks to 14 g/100 ml. at 22 weeks. The increase in haemoglobin concentration is paralleled approximately by an increase in the erythrocyte content of foetal blood.

Between the 12th and 25th weeks of gestation, the production of erythrocytes probably increases about 34 times. The count per cubic millimetre of blood doubles, while the body weight—and *pari passu* presumably the blood volume—increases seventeenfold. It is clear from these data that in the foetus erythropoiesis must be exceedingly active and stem-cell requirements correspondingly much greater than in the adult organism. The mean corpuscular volume and mean corpuscular haemoglobin fall as development proceeds but are still above the normal adult range at the end of gestation. As the mean corpuscular volume falls more rapidly than the mean corpuscular haemoglobin, the mean corpuscular haemoglobin concentration rises during foetal life. These changes in the erythrocytes may be related either to changes in the morphological site of haemopoiesis, *or* to the availability of haemopoietic raw materials or adjuvants, *or* to a combination of changes in both. Although the mean corpuscular volume of the foetal erythrocytes is initially two or three times as great as that of adult erythrocytes, the erythrocyte content of the blood is so low that the packed-cell volume is only one-third the adult value. The low packed-cell volume and the inability of foetal blood to coagulate effectively, which we have consistently observed, may be important factors in the maintenance of the extra-embryonic, placental circulation.

During the hepatic phase of foetal haemopoiesis the blood contains very few leucocytes. The sparsity of these cells accords well with the recent observation of Thomas, Russell & Yoffey (1960), that haemopoiesis in the liver is predominantly, if not exclusively, erythropoietic. As development proceeds, and the bone marrow and lymphoid tissues increase in mass, the granulocytes and lymphocytes of the blood increase in number. But, whereas the major increase in granulocyte content, like that in γ-globulin concentration, occurs in the last few weeks of gestation, the lymphocytes increase in number until the 20th week, when they attain a plateau value, which they subsequently maintain. The late maturation of the immune response together with the late increase in the granulocyte content of foetal blood and the late acquisition of γ-globulin in appreciable quantities contrast with the early attainment of the lymphocyte plateau value and it may be that in the foetus the lymphocytes are serving a non-immunological function (Yoffey & Thomas, 1961).

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Riboflavine and the haemoglobin oxidation stability of rats

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The stability of intracellular haemoglobin to oxidizing agents such as sodium nitrite can readily be determined *in vitro* by adding weak isotonic solutions of such agents to washed red cells suspended in saline. The red opalescent suspension becomes brown as the haemoglobin is converted to methaemoglobin. It has recently been shown that the increased sensitivity of newly born infants (Betke, 1952; Kunzer, Ambs & Schneider, 1953) extends throughout childhood (Keohane & Metcalf, 1960; Metcalf & Keohane, 1960), and also occurs during pregnancy and in patients with malignant disease and some other conditions (Metcalf, 1960*a*, *b*, *c*; Metcalf, 1961*a*, *b*).

A similar decreased stability occurs in the young rat (Metcalf, 1960*d*), in which it seems to be directly related to the growth rate, in the pregnant rat and following the successful transplant of the Walker Carcinoma. It was considered that this phenomenon might be due to a relative dietary deficiency as a result of the increased metabolic demands during growth, pregnancy or by the carcinoma. Starvation for 42 hr produced a similar marked decrease in the haemoglobin stability which could rapidly be restored to normal by feeding either a full normal diet or a food containing appreciable quantities of vitamins of the B group. When injected subcutaneously, the only component of this group of vitamins capable of restoring the haemoglobin stability of such starving rats was riboflavine (2 mg). Similar doses of riboflavine subcutaneously were also capable of increasing the haemoglobin stability of young and pregnant rats.

As a result of this work, it is suggested that riboflavine or its derivatives may play some part in the prevention of methaemoglobin formation *in vivo*, and that a relative riboflavine deficiency may be the explanation of the increased sensitivity to oxidizing agents of the haemoglobin of young, cancerous or pregnant rats.

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Identification of a barrier layer in the skin

By F. N. Marzulli* and R. T. Tregear. *Chemical Defence Experimental Establishment, Porton Down, Wilts*

Many compounds, especially insecticides, are toxic by the percutaneous route, but little is known of the factors involved in skin penetration. Tri-*n*-propyl phosphate (TNPP) has been used as a type substance for studying rates of penetration through excised human skin, using ^{32}P-labelled material and a diffusion chamber technique. By tests on full-thickness skin, epidermis, dermis and stripped skin (skin from which the keratin had been removed), and also on sheets of stratum corneum conjunctum (s.c.c.) obtained from volunteers, the site in the skin of a major rate-controlling barrier has been defined.

Epidermis and dermis were separated by heat (Baumberger, Suntzeff & Cowdry, 1942). Skin was stripped by successive application and removal of cellulose-backed latex tape (Sellotape), which first removed outer loose keratin (stratum corneum disjunctum) and then compact keratinous s.c.c. Sheets of s.c.c. were floated off the tape for penetration studies by petroleum ether (Szakall, 1955).

Disks of tissue, 1 cm diameter, were introduced into diffusion cells at 38° C. Two mg of TNPP was applied to a 0·2 cm² area in the centre of the disk. As the TNPP penetrated the tissue, it was swept away by isotonic saline bathing its under surface. The penetration of TNPP for up to 20 hr was estimated from the radioactivity of the effluent.

Transfer of TNPP as a function of time was qualitatively similar for full-thickness skin, stripped skin and dermis, with an initial delay before a maximum steady penetration rate was established, as Treherne (1956) reported for full-thickness rabbit skin. The high storage capacity of dermal tissue, however, masked an effect observed with isolated s.c.c. and with epidermis. Here, an initial delay was followed first by a rise, and then a fall to a steady rate; this fall may have been due to a reaction between tissue and agent which resulted in reduced tissue permeability. The delay period for all skin components was related to tissue thickness; the maximum rate through s.c.c. was reached within minutes, compared with 3 or 4 hr for full-thickness skin which was about 200 times as thick.

Steady rates of penetration of TNPP through full-thickness skin ranged from 0·4 μg/cm²/min for adult chest skin to 4·4 μg/cm²/min for the midline skin of a child. These results are higher than those reported by Tregear (1960) for the penetration of tri-*n*-butyl phosphate through excised pig skin (0·12 μg/cm²/min).

TNPP penetrated isolated s.c.c. at approximately the same rate as

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isolated epidermis, suggesting that this two- or three-cell thick (6–10 μ) continuous sheet of compact keratin provides a major barrier in skin against penetration by this organic liquid.

Removal of keratin by stripping increased the average amount of TNPP which passed in 2½ hr through full-thickness skin only 2·7 times (95 % limits 1·2–6·1) and isolated epidermis twofold (95 % limits 1·4–2·7). This suggests that there is an additional epidermal barrier below the S.C.C.

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Some possible antagonists to components of the menstrual stimulant.

By H. J. CLITHEROE and V. R. PICKLES. *Department of Physiology, University of Sheffield*

Evidence that gastrin is not a general histamine-releasing agent

By E. L. BLAIR, BRAHMA DUTT, A. A. HARPER and H. J. LAKE. *Department of Physiology, Medical School, King's College, Newcastle upon Tyne*, 1

Gastrin extracts, the acid-stimulating effects of which do not depend on the small amount of histamine they contain, can be prepared from the antral mucosa of man, pig, dog and cat (Blair, Harper, Lake, Reed & Scratcherd, 1961).

Endogenous histamine release anywhere in the body will, however, stimulate gastric secretion, and with the histamine liberator Compound 48/80 most of the released histamine appears to come from tissues other than gastric mucosa (Paton & Schachter, 1951; Smith, 1953). It remains possible that the action of these gastrin extracts might depend upon such a non-specific histamine liberation (Smith, 1954). To investigate this possibility, plasma histamine levels have been followed during acid gastric secretion in response to intravenous gastrin or histamine. Histamine was estimated by the method of Adam, Hardwick & Spencer (1957).

The experiments were performed on cats anaesthetized with chloralose and with their vagus and splanchnic nerves cut. The stomach was intubated via the oesophagus in the neck and the pylorus was occluded. Hydrochloric acid (0·005 N) was run into the stomach and replaced at intervals. Hydrogen-ion secretion was determined by titration with phenolphthalein as indicator. The cats were given heparin (700 u./kg,

intravenously) at the start of the experiment and blood was collected through a polythene tube introduced into the aorta via the carotid artery. Histamine and gastrin were given by slow intravenous injection in saline at the rate of 20 ml. over 45 min. The dose of gastrin was adjusted to produce an acid secretion comparable to that produced by the dose of histamine, which was usually about 1·6 μg/kg/min.

Histamine injected in these doses produced moderate gastric acid secretory responses, and was always associated with well marked increases in the histamine content of arterial plasma. Injections of gastrin in the same experimental animals were without significant effect upon the arterial plasma histamine levels. It is concluded therefore that the gastrin preparations used in these experiments do not depend for their effect upon a generalized histamine release. It remains possible that gastrin acts as a histamine-releasing agent locally at the gastric mucosa and this is at present under investigation.

Our thanks are due to Dr H. M. Adam for his help in enabling us to acquire the technique of histamine estimation.

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Heteronymous motoneurone activation during potentiation by high-frequency trains of impulses

By F. B. Beswick and R. T. W. L. Conroy, *Department of Physiology, University of Manchester*

Job (1953) and Beswick & Evanson (1954) showed that heteronymous motoneurones of synergistic muscles were activated on stimulating large afferent muscle nerve fibres during post-tetanic potentiation. This effect was demonstrated after 30 sec tetanization of the Group Ia fibres at frequencies about 600 c/s.

An investigation of the activation of heteronymous motoneurones has been made using trains of high frequency stimuli of 1 sec duration, repeated every 5 sec. These were applied to one ankle extensor muscle nerve in the hind limb of the decerebrate cat. Single testing shocks were applied to the same nerve almost 4 sec after the end of each train. Records of the discharges of heteronymous motoneurones were taken from the nerve of a synergistic muscle. Such records have been made using the nerves to

the medial and lateral heads of gastrocnemius, plantaris and the deep posterior muscles of the leg, in various combinations of pairs.

On testing, repetition of the trains of volleys caused the appearance of monosynaptic reflex action potentials in the unstimulated nerve. The responses gradually increased to reach maximum size between 10 and 20 min after the commencement of conditioning. The optimum frequency for the trains was found in these experiments to lie between 800 and 900 c/s.

The maximum response could be maintained by repeated trains of volleys for as long as 2 hr. If, however, conditioning by a continuous 1 min tetanus delivering the same number of stimuli in the minute was substituted, subsequent testing resulted in submaximal reflex discharges. If the frequency of the continuous tetanus were increased from 200 to 800 c/s (approx.), the subsequent testing response remained submaximal.

It seems, therefore, that continuous tetanic stimulation may evoke less potentiation than repeated trains of stimuli.

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Grouping of action potentials in voluntary muscle

By A. Taylor. *Department of Physiology, King's College, London, W.C.2*

Action potentials of electromyograms recorded from steadily contracting voluntary muscle often appear to be grouped at intervals of about 0·1 sec. This grouping has been shown by Lippold, Redfearn & Vuco (1957) to underlie the prominent 10 c/s component of physiological tremor, which these authors attributed to oscillation in stretch reflex arcs.

Observations of the electromyogram of the diaphragm in conscious human subjects and in anaesthetized or decerebrate rabbits and cats has shown a similar appearance of grouping in all cases. However, grouping persisted in the animals' diaphragms after their deafferentation by cutting all the cervical posterior nerve roots and the cord immediately below the phrenic outflow after vagotomy. Grouping of action potentials was also present in phrenic nerve slips of curarized decerebrate cats. The grouping could not then have been due to stretch reflex oscillation.

Records from an electronic model showed that a number of independent units firing regularly, or with some random scatter, could produce a similar appearance of grouping.

It is suggested that this type of chance grouping could contribute to mechanical tremor. It could only be distinguished from a true tendency to

synchronization of motor unit activity by detailed statistical analysis of the records.

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Acclimatization of the sweating mechanism in man

By R. H. FOX, R. GOLDSMITH, D. J. KIDD and H. E. LEWIS. *Division of Human Physiology, National Institute for Medical Research, London, N.W.3*

The development of acclimatization to heat in man, as judged by an increase in the rate of sweat loss on exposure to a standard heat stress, is probably related both to the intensity and to the duration of the imposed heat stress. In the present investigation the relative effectiveness of three levels of each of these two parameters have been studied. Eighteen fit young men were randomly assigned to nine treatment groups comprising three body temperature levels (37·3, 37·9, 38·5° C) and three durations of exposure ($\frac{1}{2}$, 1, 2 hr). The acclimatization period consisted of twelve daily exposures. Body temperature was first rapidly raised to the target level in a sauna-like climate (D.B. 43° C, W.B. 40·5° C, air speed approximately 200 ft. (61 m) min). The subject was then totally enclosed, except for the face, in a vapour barrier suit, proceeded to a further room (D.B. 38° C, W.B. 26° C, air speed 50 ft./min) and reclined throughout his particular duration of exposure. Body temperature was monitored continuously by indwelling oral thermistors and maintained on target by controlled ventilation of the vapour barrier suit. Before and again after the acclimatization period the subjects were given a 2-hr uniformity test comprising controlled activity in a standard hot climate (D.B. 40° C, W.B. 32° C, air speed 50 ft./min).

During the acclimatization period the subjects maintained at the lowest body temperature level did not show an increase in sweat rate, whereas those maintained at the highest body temperature level exhibited a progressive increase, which was most marked for the subjects with the longest exposure (120%) and least for those with the shortest exposure (50%).

The uniformity tests showed that acclimatization was enhanced both by increasing the duration of exposure ($P > 0{\cdot}05$) and by increasing body temperature ($P > 0{\cdot}01$). Even the subjects maintained at 37·3° C for $\frac{1}{2}$ hr exhibited a significant acclimatization effect.

Pulse rate responses showed little change, suggesting that this type of heat exposure, in which physical activity is at a minimum, is relatively ineffective in improving the cardiovascular response to heat.

It is concluded that quite small elevations of body temperature, maintained for short periods daily in the manner described, are capable of

causing an acclimatization of the sweating mechanism in man, and that this effect is markedly potentiated both by increasing the degree of elevation of body temperature and the duration for which it is elevated each day.

Changes in peripheral blood flow with heat acclimatization

By R. H. Fox, R. Goldsmith, D. J. Kidd and H. E. Lewis. *Division of Human Physiology, National Institute for Medical Research, London, N.W.3*

While investigating the effect on the sweating mechanism produced by twelve daily elevations of body temperature to three different levels (37·3, 37·9 and 38·5° C) and maintained for three different durations ($\frac{1}{2}$, 1 and 2 hr), the changes in the peripheral blood-flow response to body heating were also examined. The nine male subjects, each representing one of the

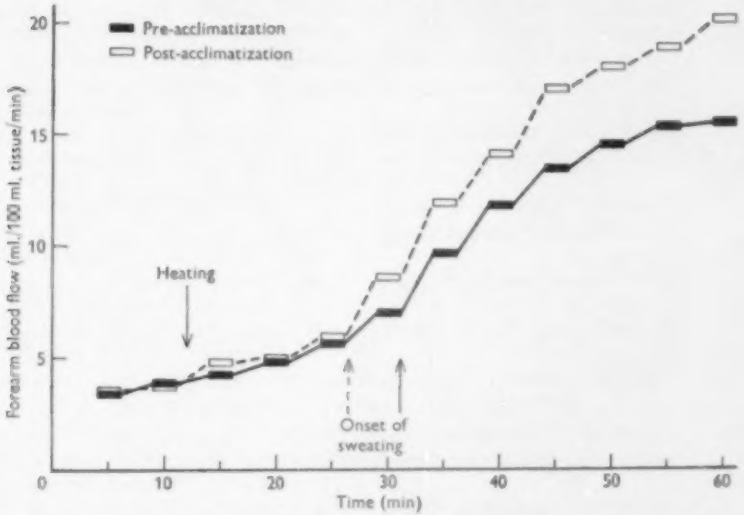

Fig. 1. Mean forearm blood flows and onsets of sweating for eight subjects before and after acclimatization, showing the response to body heating.

treatment combinations, were tested before and again after acclimatization. The subjects sat in a stirred water-bath which was slowly heated from 34° C by a constant inflow of water at 43° C until the subject's oral temperature reached 38·5° C. Blood flows in the hand and forearm were measured by plethysmographs and changes in the skin of the chest and pinna of one ear by surface calorimeters. Body temperatures were continuously monitored by indwelling oral thermistors. Water losses from

chest and forearm skin were followed with ventilated capsules and hygrometers. Each subject's body temperature curves before and after acclimatization were almost identical. Both the time from heating to the onset of sweating and the rate of vasodilatation in the forearm when sweating started varied quite widely from individual to individual, as might be expected. Mean forearm blood flows for eight subjects, omitting one with incomplete data, before and after acclimatization, and the times of onset of sweating, are illustrated in Fig. 1. The earlier onset of sweating for individuals after acclimatization ($P < 0{\cdot}01$) agrees with the observation of Ladell (1947). Blood flow at the end of the heating period was higher after acclimatization in both forearm ($P < 0{\cdot}02$) and hand ($P < 0{\cdot}01$) and a similar trend was also present in the chest and ear.

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